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SPECIAL ARTICLE

Guidelines for HER2 testing in breast cancer: a national consensus of the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM)

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Abstract Identifying breast cancers with HER2 overexpression or amplification is critical as these usually imply the use of HER2-targeted therapies. DNA (amplification) and protein (overexpression) HER2 abnormalities usually occur simultaneously and both *in situ* hybridisation and immunohistochemistry may be accurate methods for the evaluation of these abnormalities. However, recent studies, including those conducted by the Association for Quality Assurance of the Spanish Society of Pathology, as well as the experience of a number of HER2 testing National Reference Centres have suggested the existence of serious reproducibility issues with both techniques. To address this issue, a joint committee from the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM) was established to review the HER2 testing guidelines. Consensus recommendations are based not only on the panellists' experience, but also on previous consensus guidelines from several countries, including the USA, the UK and Canada. These guidelines include the minimal requirements that pathology departments should fulfil in order to guarantee proper HER2 testing in breast cancer. Pathology laboratories not fulfilling these standards should make an effort to meet them and, until then, are highly encouraged to submit to reference laboratories breast cancer samples for which HER2 determination has clinical implications for the patients.

Keywords Breast cancer · HER2 · Immunohistochemistry · *In situ* hybridisation · Standardisation · Quality assurance

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Introduction

Clinical relevance of HER2 testing in breast cancer

The human epidermal growth factor receptor 2 gene c-*erbB*-2 (commonly referred to as *HER2/neu*) is located at the long arm of chromosome 17 and encodes the HER2 protein, a transmembrane receptor with tyrosine kinase activity [1]. HER2 belongs to the epidermal growth factor receptor (EGFR) family, also known as the HER family. This family includes four members (HER1 to HER4) and, under physiologic conditions, plays a role in intercellular and cell–stromal communication [2, 3].

However, HER receptors show an abnormal signalling activity in a wide range of tumours. Within this family, HER2 is particularly oncogenic [4]. The following evi-

dence leads to the consideration of HER2 as a therapeutic target: HER2 gene transfection induces a malignant phenotype; HER2 is overexpressed in 17–20% of human breast cancers; the primary cause for HER2 overexpression is gene amplification; HER2 overexpression or gene amplification leads to a poor prognosis of patients with breast cancer [5]; and, finally, in the late 1990s it was demonstrated that monoclonal antibodies targeting HER2 were able to produce anti-tumour effects. Among these antibodies, the murine 4D5 antibody was particularly active in cell lines or tumours overexpressing the HER2 protein. The humanisation of the 4D5 resulted in the anti-HER2 antibody trastuzumab (Herceptin, Roche, Bassel) [6].

In the last decade, HER2 testing has become a routine test in the evaluation of invasive breast cancer. However, the best method for HER2 evaluation and even diagnostic algorithms is still controversial. This is an important issue to clarify, due to the influence of HER2 testing in clinical decision-making at two levels, prognosis and prediction of response. Relative to prognosis, HER2 status, among other factors, may affect the decision of administering adjuvant therapy to women with breast cancer. Furthermore, information on the HER2 status is crucial to decide the use of anti-HER2 therapy. Several studies have demonstrated the great efficacy of the monoclonal anti-HER2 antibody trastuzumab for the treatment of all stages of breast cancer [7, 8]. Trastuzumab, administered with chemotherapy, increased the response rate, the progression-free interval and the survival of patients with HER2-positive metastatic breast cancer, also being active when administered in monotherapy. Pivotal studies included patients with HER2-positive tumours, with levels of expression of HER2 protein of 2+ and 3+ [9, 10]. Retrospective analyses have suggested that only those patients with 3+ staining and/or HER2 gene amplification by fluorescent *in situ* hybridisation (FISH) benefited from trastuzumab therapy [11].

Remarkably, the use of trastuzumab in the adjuvant setting (i.e., postoperatively) in patients with HER2-positive early breast cancer reduced the risk of relapse by half and the risk of mortality by a third. These results were supported by five phase III multicentric studies including over 13,000 females [12–16]. All trials of adjuvant trastuzumab included patients with invasive, HER2-positive tumours (either 3+ staining by immunohistochemistry or FISH amplification). The HER2 status was determined by chromogenic *in situ* hybridisation (CISH) in one of these trials. Overall, the five studies indicated methodological differences in HER2 status testing, reinforcing again the need for standardisation of this technique and its evaluation [7, 8]. Besides its efficacy in metastatic breast cancer and as an adjuvant therapy, the addition of trastuzumab to chemotherapy in the neoadjuvant setting (i.e., preoperatively) in HER2-positive tumours resulted in a three-fold increase of complete remission rates [17]. The addition of lapatinib (Tykerb, GSK), a dual inhibitor of the HER1/HER2 tyrosine kinase activity, combined with capecitabine, has been recently reported to improve the clinical outcome of patients with HER2-positive advanced breast cancer after progression with chemotherapy plus trastuzumab [18]. The use of trastuzumab plus capecitabine has also been shown to be more efficacious than capecitabine alone, after progression with chemotherapy and trastuzumab [19].

Though it is generally well tolerated, anti-HER2 therapy is associated with several adverse side effects, particularly a clinically relevant risk of heart toxicity. Thus, at median follow-up times of three years or less, between 5% and 15% of patients developed cardiac dysfunction, and between 1% and 4% of patients showed significant cardiac events [20, 21]. Furthermore, although treatment duration varies widely, currently adjuvant trastuzumab therapy is recommended for 12 months.

Current status of HER2 testing in Spain: the experience of the SEAP Quality Assurance Program

The SEAP's Association for Pathology Quality Assurance (AGCP) was established in January 2004 with the purpose of encouraging and promoting the control of the quality of care and research in pathology laboratories. During the year 2004, Surgical Pathology and HER2 modules of the Immunohistochemistry Quality Assurance Program were initiated, while the Breast Pathology and Lymphoid Tissue modules were initiated in 2005.

Participant laboratories receive blank programme preparations to carry out the requested immunohistochemical (IHC) techniques and they returned these preparations together with control preparations. A committee consisting of four experts evaluated all preparations. The Program guarantees anonymous participation and confidential disclosure of results. At least 125 centres participated in the data analysis from the HER2 module of the SEAP Quality Assurance Program, conducted from October 2004 to May 2008 with a total of 8 rounds, although only 8 centres took part in all rounds (6.4%). Participation data indicated that 64 centres (62.4%) underwent only between 1 and 4 rounds.

Different evaluation rounds were conducted on different levels of HER2 protein expression tissue samples. All samples were fixed for 24 h in 10% buffered formalin at a pH of 7.0 and subsequently embedded in paraffin. Evaluation criteria were the same in all rounds. Optimal results were assigned to those cases with correct results and no tissue artefacts in all study samples, allowing appropriate evaluation of these cases. Acceptable results were assigned to those cases allowing the evaluation, but with decreased or increased intensity in some sections, that in certain cases might result in a deficient interpretation of the results. Inadequate results were assigned to those cases in which the results were incorrect, due to an absence of expression in 3+ and 2+ cases, possibly related to insufficient antigenic retrieval or use of a low sensitivity technique; or, on the other hand, marked overexpression in negative cases (0 and 1+), frequently related to an intense and diffuse expression

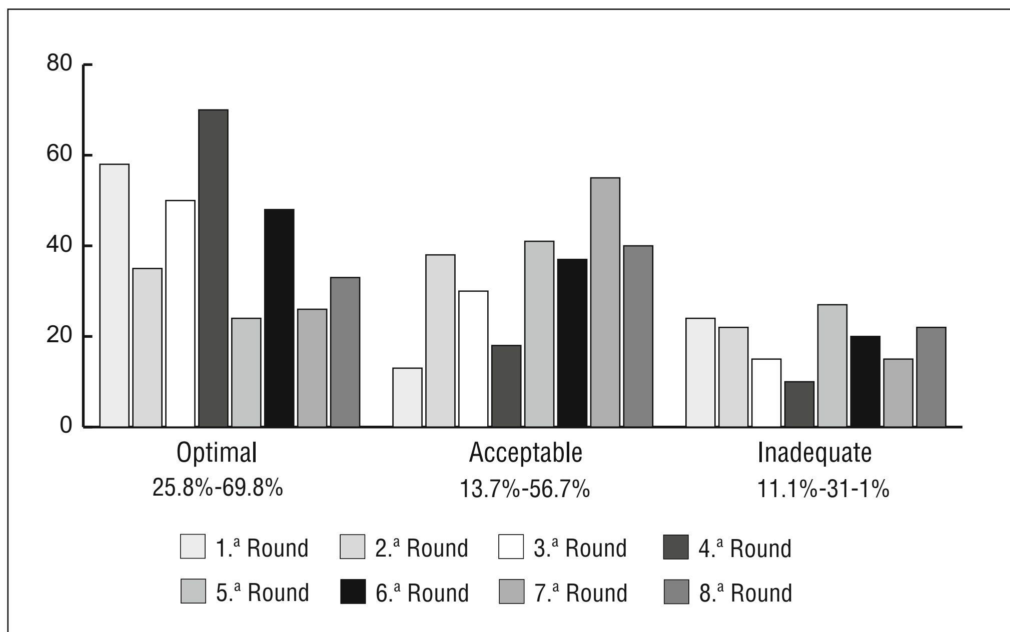

Fig. 1 Comparative results of the 8 rounds of HER2 IHC module of the SEAP Quality Assurance Program

in normal ducts and, on occasion, to an unspecific staining of the stromal cells, resulting in erroneous interpretations.

Analysis of the results of the 8 centres that participated in the 8 rounds indicated optimal and acceptable results with no inadequate results in 4 centres (50%), although only one centre obtained optimal results in all rounds; the remaining 4 centres obtained inadequate results in one or more rounds. None of the centres participating in 4–7 rounds (53 centres) obtained optimal results in all rounds in which they participated and only 13 centres (24.5%) obtained optimal and acceptable results with no inadequate results. Out of 64 centres that took part in 1–3 rounds, 15 (23.4%) obtained optimal results in all rounds, and 39 centres (60.9 %) obtained optimal and acceptable results with no inadequate results.

In summary, and depending on the rounds, the percentage of optimal scores ranged from 25.8% to 69.8%; from 13.7% to 56.7% for acceptable results; and from 11.1% to 31.1% for inadequate results (Fig. 1). The use of FDA-approved marketed kits (HercepTest and Pathway) progressively increased throughout the different rounds and attained a percentage of 64.9% by the 8th round.

A total of 47 centres participated in the FISH/CISH module, although only one centre underwent the 8 rounds (2.1%); 12 centres participated in 4–7 rounds (25.5%); and 34 centres in 1–3 rounds (72.4%). Problem preparations containing various tissue sections with different levels of HER2 amplification were assessed. Tissues had been fixed for 24 h in 10% buffered formalin solutions at a pH of 7.0 and subsequently embedded in paraffin. The same evaluation criteria were used in all rounds. Optimal results were assigned to study samples with correct results and no tissue artefacts, allowing a proper interpretation. Acceptable results were assigned to study samples, allowing evaluation but with increased intensity of probes in some sections, which in certain cases might result in a deficient evaluation. Inadequate results were assigned to those samples in which the results were incorrect, primarily due to excessive digestion; a marked unspecific background hybridisation, leading to erroneous readings; or inadequate material for evaluation.

Thirteen centres participated in 4–8 evaluation rounds of FISH/CISH, and 7 centres obtained optimal and acceptable results with no inadequate results (53.8%). Out of 34 centres that participated in 1–3 rounds, 29 obtained optimal and acceptable results with no inadequate results (85.2%). Fifteen centres (31.9%), out of a total of 47, obtained optimal results in all rounds in which they participated.

In summary, depending on the rounds, optimal results ranged from 16.7% to 85.7%; acceptable results from 14.3 to 66.6%; and inadequate results from 11.1% to 21% (Fig. 2).

Current status of HER2 testing in Spain: the experience of Reference Centres

Between 2001 and 2008, Reference Laboratories were established through an agreement between SEAP and Roche Farma S.A. These centres were initially planned to conduct FISH analysis in breast carcinoma samples with an IHC result of +2, with the aim of selecting patients sensitive to trastuzumab treatment.

In spite of differences in the number of studies and organisation, all centres had similar methods, endpoints and results. Initially, the Reference Laboratories found important discordances in the results between IHC and FISH

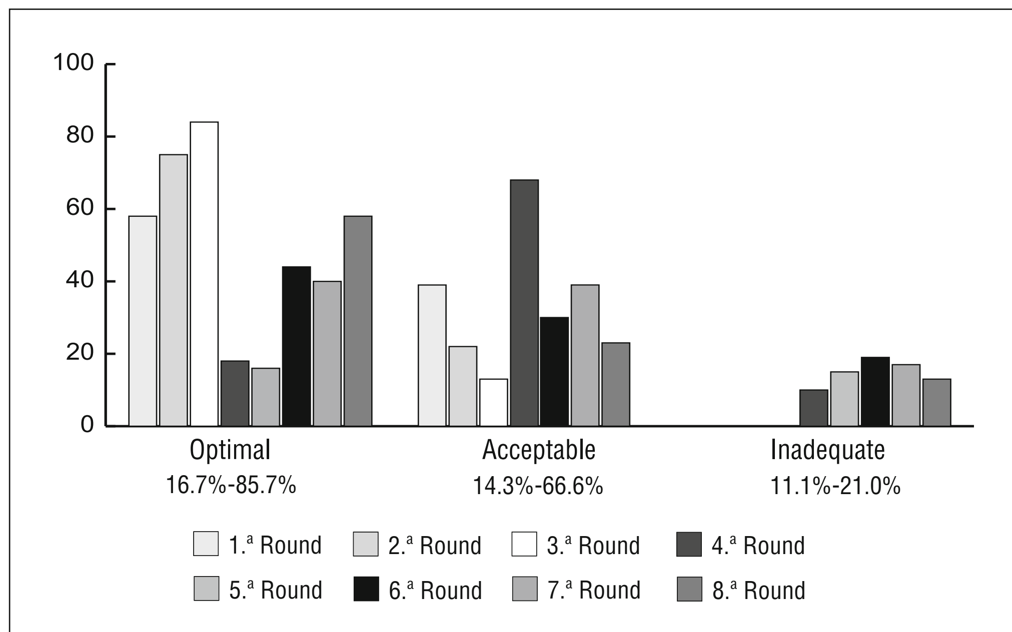

Fig. 2 Comparative results of the 8 rounds of HER2 FISH/CISH module of the SEAP Quality Assurance Program

analysis. There were different reasons for this, including lack of procedure and technique normalisation, variability in the evaluation of samples and, particularly, suboptimal tissue quality due to inadequate fixation, processing and storage conditions.

Since these studies were of a retrospective nature and included cases of patients with metastatic disease with poor response to conventional therapies, paraffin blocks were old, and this could be a source of bias. During this first part of the study, a high percentage of false positive and false negative cases were observed (considering FISH as the gold standard). Almost 50% of the analysed tumours showed HER2 gene amplification and the level of aneusomy of chromosome 17 was also high, probably due to the biased patient selection.

Regardless of the significant progress of technique normalisation and the acquired experience in IHC reading, this problem was gradually solved afterwards, when new cases were studied. Due to the initially observed discordances, Reference Centres began to repeat IHC studies simultaneously to FISH studies with a level of concordance under 60% between both techniques during this first phase of the study. These figures have gradually improved, but concordance levels between IHC and FISH are still under 80%, which appears to be rather low. However, complexity, evaluation difficulties and equivocal levels of expression of cases selected for FISH should be considered. In fact, not only 2+ cases are actually submitted for FISH analysis, but also those presenting assessment problems of a different nature.

A trend towards obtaining slightly worse results in Reference Laboratories when IHC testing is done on samples submitted for FISH study should be highlighted, although all possible combinations may be observed. This might be partially due to submission of cases with strong or borderline 1+ stain pattern, which have been directly considered equivocal 2+ or 3+ in order to request a consultation. Overall, during this period nearly 12,000 tumours were analysed. In spite of the heterogeneous and biased sample population, most cases matched primary infiltrative breast carcinomas, usually of the ductal type. However, biopsies of recurrent tumours, and lymph node, visceral and even bone metastasis were included. Occasionally, more than one sample from the same patient was studied, and this allowed the observation, in some cases, of differences in the staining pattern between different areas of the same tumour, or between a primary tumour and its recurrence/metastasis. This phenomenon (although rare) was more frequently observed in IHC than in FISH studies. Approximately 50% of cases submitted to Reference Laboratories for IHC studies were considered positive (2+/3+) and nearly 25% showed HER-2 amplification. Negative tumours (0/1+) with gene amplification were also observed, although these were exceptional cases.

The following general conclusions may be pointed out: HER2 testing exhibits notable reproducibility difficulties; IHC has become a common method of choice in daily routine, but HIS (Hybridization *in situ*) analysis has been recommended for uncertain cases, or when the quality or particularities of the sample makes proper assessment difficult; the observed inter/intra-institutional variability is mostly due to technical, handling or interpretation issues, particularly with IHC, but also with FISH. This is more evident in cases of low gene amplification; laboratories with

specifically devoted pathologists responsible for HER2 testing usually report greater correlation rates between IHC and FISH results.

Due to the aforementioned observations, Reference Laboratories have stressed the importance for Pathology Departments to participate in Quality Assurance Programs, such as the SEAP program, for diagnosis level monitoring and participation in training programmes, both essential if accreditation programmes are to be developed for centres and professionals. Centres should establish a follow-up programme for diagnosed cases with the aim of identifying incidences or variations, and take the appropriate corrective measures.

Need of a consensus guideline for HER2 testing

On the basis of all previous observations, it seems obvious that a reliable HER2 status testing of patients with breast cancer is an essential requirement for an appropriate use of anti-HER2 treatment, as well as for prognosis assessment. Thus, ideally HER2 testing should have 100% sensitivity and 100% specificity. However, in clinical practice, HER2 testing poses many difficulties [7, 8]. Various studies, including our own SEAP data, evidenced the distance between reality and this objective [22, 23]. Furthermore, with the increased knowledge of HER2-positive breast cancer, new clinically relevant questions appear. Among them, the switch of initially HER2-negative tumours to HER2-positive tumours during relapse, the interpretation of chromosome 17 polysomy and changes in HER2 expression induced by neoadjuvant therapies should be emphasised as particularly relevant examples [24]. Thus, several countries have developed consensus guidelines or recommendations in order to improve HER2 testing accuracy.

Basically, three factors encouraged us to develop this National Consensus Guideline between the Spanish Society of Pathology (SEAP) and the Spanish Society of Medical Oncology (SEOM). Firstly, the SEAP has been actively working with pathologists training on HER2 testing, and studies of concordance have been conducted among different pathology laboratories (volunteers) against national reference laboratories. After several rounds, results showed significantly lower concordance rates in many cases. Thus, new strategies should be developed to achieve reproducible HER2 testing in our country, i.e., to avoid the quality heterogeneity of current testing. Secondly, the results of anti-HER2 therapies in breast cancer and, mainly, the generalised use of adjuvant trastuzumab in HER2-positive breast cancer in our country, result in an even greater need for regulation of HER2 testing. Thirdly, there is a need for setting in a context international guidelines, and new assessment models for HER2 [7, 8, 25, 26].

For these reasons, a panel of pathologists with expertise in HER2 testing together with medical oncologists with dedication to breast cancer, on behalf of their respective societies (SEAP and SEOM), joined to discuss this issue in order to reach a national consensus on the recommendations for HER2 testing. These recommendations were not only based on the participants' experience, but also on international experience, published in recent consensus guidelines from different countries, included the USA [7, 8], UK [27] and Canada [24].

The outcome of this consensus was these guidelines, developed to be used by pathologists and clinicians from our country in daily practice. The guideline has a bidirectional objective. Firstly, to serve as continuous training and stimulus for pathologists to follow the established recommendations and to develop internal validation models as well as an external SEAP accreditation. Laboratories not fulfilling the minimum standards described in this guideline should try to adopt these standards and, meanwhile, submit those samples in which HER2 testing may have clinical implications to reference laboratories. Secondly, to promote knowledge on the difficulties of HER2 testing and the medical oncologist interpretation. This might result in improved joint analysis of results, as well as the oncologist's awareness of whether the HER2 results he/she receives were obtained in accordance with the minimum acceptable standards proposed in this guideline.

Recommended conditions for HER2 testing

Testing time

Current clinical practice requires HER2 testing of all patients with infiltrative breast cancer due to both prognostic and predictive values (Table 1). Thus, HER2 should always be tested before treatment indication in early breast cancer as well as in advanced or metastatic breast cancer. In the early stages of breast cancer, HER2 testing of the primary tumour is the rule, and concordance levels of at least 90% have been published between HER2 status of primary tumours and their paired metastasis [28]. Changes from HER2-negative primary tumours to HER2-positive relapsed tumours have been documented, particularly in patients receiving hormone therapy. Since anti-HER2 treatment should be considered in such cases, patients with local or metastatic relapse and available tissue samples from a biopsy or a surgical resection should undergo new HER2 testing. The practice of biopsies of metastatic disease, with the purpose of checking biomarkers predicting the response status (HER2, hormone receptors) at the time of therapeutic decision-making, has become increasingly important [29, 30].

On the other hand, HER2 negativisation cases have been observed among HER2+ breast cancer patients treated with trastuzumab [29]. The significance of this change is still unknown. Since the existence of a micrometastatic disease with a different HER2 status (i.e., HER2 positive) cannot be excluded, decision-making on anti-HER2 therapy based on this test does not seem advisable with the current data. We expect that, in the future, we will know patients'

Table 1 HER2 testing in breast cancer

Indication
All patients with a diagnosis of invasive breast cancer (early, advanced or metastatic) before treatment initiation

Type of sample
Biopsy or surgical specimen from the primary tumour.
In case of relapse or metastasis, the use of a sample from the relapsed or metastatic lesion is recommended, if available
The use of *in situ* hybridisation techniques is recommended for suboptimal samples (fixation defects, retraction artefacts, crushing, cauterisation, etc.)
Cytologic samples are only recommended for *in situ* hybridisation studies, provided no alternative tissue samples are available

HER2 status during relapse, and this will show whether the changes that appeared during neoadjuvant therapy correlate with the relapsed disease phenotype.

Type of samples

Both biopsy (BAG, BAV) and surgically obtained samples are appropriate for IHC and/or *in situ* hybridisation (HIS) HER2 testing, provided they include an infiltrative component of the tumour, preferably far from the *in situ* component.

In cases of surgically obtained samples (mastectomy, tumorectomy, etc.) an appropriate macroscopic study should always include the tumour size, margins and orientation. Samples should be sliced at 5-mm intervals to facilitate fixation under optimal conditions. Certain technical artefacts such as retraction, crushing artefacts, marked cauterisation, etc., are more frequently observed in biopsy-obtained samples, while fixation problems are more common among surgical resection samples (particularly from a mastectomy). The use of *in situ* hybridisation techniques is recommended for suboptimal sample testing for these reasons. Cytologic samples are only recommended for *in situ* hybridisation studies, provided alternative tissue samples are unavailable.

Fixation

For all samples, it is recommended to record the time elapsed to fixation, the fixation time and the fixative employed [7, 8] (Table 2). The interval between tissue acquisition and fixation of breast specimens should be as short as possible. Incisional and excisional biopsy samples used for HER2 testing should be fixed in 10% neutral buffered formalin for intervals ranging from 24 h to no more than 48 h. The use of a volume of at least 4-fold the volume of the specimen is recommended [31]. Alternative fixatives should not be used, since alcohol-based fixatives (Z-5, Pen-Fix) may result in false positive results on IHC and HIS testing. Furthermore, Bouin or Zenker fixatives might preclude a subsequent HIS study. In HER2 testing, a fixation deficiency is considered more critical than an excessive fixation time. Small samples fixed under 6 h should not be used for IHC or HIS studies. Prolonged fixation (more than 48 h) or underfixation (under 24 h) may result in false-negative results. Rapid tissue processing protocols based on the use of microwaves are not recommended. The use of decalcified tissues for HER2 testing has not been validated and, therefore, it is not recommended.

Paraffin embedment and microtomy

Tissue sections should be 1–1.5 cm length and 0.2 cm thick for proper processing. Paraffin blocks should be stored at room temperature (20–25ºC). Samples should be routinely processed into paraffin and cut onto glass treated slides to prevent detaching during the subsequent processing within 48 h. Sections should be 3–5 µm thick. Before HER2 testing, it is recommended to dry the slides at 60ºC for 1 h or

Table 2 Fixation and processing conditions for HER2 testing in breast cancer

Samples should be fixed as soon as possible, in a neutral buffered 10% formalin solution, always within the first hour after sample collection
No alcohol based (Z-5, Pen-Fix) or mercury-containing (Bouin, Zenker) fixatives should be used
Rapid fixation methods, such as those based on the use of microwaves, are not recommended
The optimal fixation time is between 24 and 48 h
Samples from specimens fixed under 24 h or over 48 h may result in false-negative results
Small samples (BAG) should be fixed for at least 6 h
Do not use small samples fixed under 6 h
Sections stored over 6 weeks should not be used for IHC studies and sections stored over 6 months should not be used for *in situ* hybridisation studies
If sample testing is delayed over two weeks after slide preparation, storage of previously paraffined sections is advisable
The use of *in situ* hybridisation techniques is recommended for suboptimal samples

Table 3 Exclusion criteria to perform or interpret an HER2 assay in breast cancer

The recommended pre-analytical (fixation) and analytical (microtomy, immunostaining) requirements are not attained
Unsatisfactory control results
Absence of an infiltrative component in the slide
Samples with only limited invasive carcinoma (microinfiltrative carcinoma) difficult to evaluate under the fluorescence microscope (<20 cells)[a]
Strong and extensive membrane staining of normal ducts or acini (a weak and focal stain does not exclude or modify the interpretation of results)[b]

[a]For the *in situ* hybridisation technique; [b]for the IHC technique

at 37ºC overnight. Ideally, sections stored for more than 6 weeks should not be used for IHC HER2 testing, and sections stored more than 6 months should not be used for *in situ* hybridisation HER2 testing, due to a greater likelihood of false-negative results in both cases. If sample testing is foreseen to occur more than two weeks after slide preparation, it is recommended to embed the slides in paraffin before storage. In this case, processing of samples for HER2 testing will require a more exhaustive deparaffining process (twice as long as usual).

Recommendations for immunohistochemical HER2 testing

Method

The use of FDA- and/or European Agency-certified diagnostic kits previously validated in the laboratory is recommended. Validation may be accomplished with 25 positive and negative cases, checking the results against a reference laboratory. The use of standardised kits requires the strict fulfilment of the manufacturer's instructions, without any modification. The use of nonstandardised methods requires a more strict initial validation. At least 50 cases will be used, half of which should be unequivocally positive and the other half negative. The level of concordance with the reference laboratory should be at least 95%. Any change in the method will require a new validation. Grouping cases for testing (at least 4) is advisable, in order to have sufficient available cell-line controls.

The optimal number of annual cases reported to guarantee the laboratory's technical sufficiency is 250.

Controls

Control cell-lines provided with the kit should be used with each batch of tests. The use of own controls with the same laboratory's fixation and processing conditions is also recommended. A weak–moderate immunostaining (2+) case should be used to allow easy detection of mild sensitivity losses. The use of an appropriately validated control is compulsory if control cell lines are not available. And the own laboratory methods are used.

Exclusion criteria to perform or interpret an HER2 IHC assay

A test will be considered inappropriate for HER2 expression assessment when the mentioned pre-analytical (fixation) and analytical (microtomy, immunostaining) minimum requirements are not attained (Table 3). Additionally, a test will be rejected in any of the following circumstances: absence of an infiltrative component of the tumour in the slide; and powerful and extensive membrane stain of normal ducts or acini (a weak and focal stain does not exclude the validity of the test).

Interpretation criteria

A pathologist will be responsible for the interpretation of results. Interpretation of results should exclusively be based on the infiltrative component of the tumour and only membrane staining should be evaluated (Table 4). A negative HER2 test is defined as an IHC result of 0 or 1+ for cellular membrane protein expression (no staining or weak, incomplete membrane staining in <10% of cells (0),

Table 4 Interpretation criteria of IHC HER2 testing

Only the infiltrative component of the tumour and membrane staining should be evaluate.
Interpretation criteria:
Negative (0): absence of membrane staining or <10% of stained cells
Negative (1+): weak and partial membrane staining in >10% of cells
Equivocal (2+)[a]: weak or moderate complete membrane staining in >10% of cells or complete and strong staining in 10–30% of cells
Positive (3+): complete and strong membrane staining in >30% of cells

[a]In order to indicate the additional study using *in situ* hybridisation, those cases with interpretation difficulties, due to minor fixation artEfacts, mild over-unmasking with discrete stain of normal breast epithelium, or strong but partial membrane staining, are included in the group (2+), although they are not specifically mentioned in international guidelines

Table 5 Reporting elements for IHC HER2 testing

Patient identification information
Physician identification
Date of service
Specimen identification (case and block number)
Type of sample and anatomic origin
Fixative type (compulsory), time to fixation (recommendable) and duration of fixation (recommendable)
Antibody and method (clone, supplier, specify whether it is approved by the FDA or by any other regulatory agency)
Method used (semi-quantitative, image analysis)
Adequacy of sample for evaluation (appropriate/inappropriate for diagnosis)
Interpretation of results:
Positive (3+)
Borderline (2+)
Negative (0/1)
Not interpretable

or weak or partial membrane stain in >10% of cells (1+)). A positive HER2 test is defined as an IHC result of 3+ cell surface protein expression (defined as uniform intense membrane staining of >30% of invasive tumour cells) [7, 8]. An equivocal result (2+) is complete membrane staining that is either nonuniform or weak in intensity in >10% of cells, or complete and powerful stain in 10–30% of cells.

It should be emphasised that when most cells show partial membrane staining, but complete staining of cells is observed in >10% and <30% of cells, a result of 2+ will be assigned. This group (2+) should also include those cases with difficulties in interpretation due to minor fixation artefacts, mild over-unmasking with discrete normal breast epithelium stain or intense but incomplete membrane staining. As a general rule, in case of uncertainty on the suitability of the IHC technique, the HIS technique will be employed. If a positive result is obtained in well differentiated carcinomas [32], mucinous, tubular or lobular carcinomas (except the pleomorphic variant of lobular carcinoma), or a negative result is obtained in Paget's disease or inflammatory carcinoma [33], the results should be reviewed.

At the end of the year, laboratories should calculate the percentage of cases corresponding to each score (0, 1+, 2+ and 3+), in order to confirm whether their results are adjusted to those obtained in large series. In this sense, a review conducted in the UK on over 17,000 cases from 2007 demonstrated the following mean percentages for the different expression categories: 41% (0), 28% (1+), 20% (2+) and 11% (3+) [27]. Furthermore, amplification was observed in 19% of 2+ cases, meaning that 15% of breast carcinomas were eligible for treatment with trastuzumab. Greater percentages of positive cases were published in previous series, mostly due to the great number of metastatic breast carcinomas included in these series, which probably is the reason for such high rates of HER2-positive carcinomas.

Staff

In order to guarantee expertise, the number of laboratory technicians conducting the test, as well as pathologists interpreting the test, should as low as possible. Both, technicians and pathologists must undergo a training period. They should periodically validate their training in specific working sessions.

Report

The final report can be adapted to the information system used in each hospital. The specific list of elements recommended to be included in the final report is provided in Table 5. The recording of the specific stain pattern in the final report is not essential, since this information is implicit in the final interpretation of results. It is recommended to report the presence of protein expression heterogeneity, at least in those cases with strong and complete membrane staining in <30% of tumour cells (2+ cases). It is recommended to clearly state in the report whether the method has been approved by the FDA, and whether manufacturers' recommendations have been strictly fulfilled. In case of using a method not approved by the FDA or an FDA-approved method modified by the laboratory the report should clearly indicate what changes have been appropriately validated. The inclusion of a note in the report indicating, when applicable, that the laboratory participates in external quality assurance programmes (SEAP, UK NEQAS, NordiQC, etc.), or the laboratory has been certified or approved for the procedures relative to HER2 testing, is also recommended.

Recommendations for *in situ* hybridisation HER2 testing

Method

The use of FDA- and/or the European Agency-certified diagnostic kits previously validated in the laboratory is recommended. Validation may be accomplished with 25 positive and negative cases, checking the results against

Table 6 Interpretation criteria for in situ hybridisation HER2 testing

Interpretation criteria
Only the infiltrative component of the tumour should be evaluated
At least 20 cells will be analysed of at least two different tumour areas
Interpretation (double probe techniques):
Non-amplified: HER2 gene signals/chromosome 17 signals ratio <1.8
Amplified: HER2 gene signals/chromosome 17 signals ratio >2.2
Equivocal: HER2 gene signals/chromosome 17 signals ratio 1.8–2.2
Polysomy: Number of centromere 17 signals per nucleus >3
Monosomy: Number of centromere 17 signals per nucleus <1.5[a]
Not interpretable: Occurrence of at least one of the following circumstances:
Absence of signals from either of the probes in at least 20 cells
Weak or nonexistent signals in >25% of cells
Analysis of at least two different areas of infiltrative carcinoma is not possible
Controls do not show the expected result

[a]In some cases of monosomy 17, the HER2/CEP17 ratio=2 is determined by the presence of a single copy of the centromere 17, and two copies of the HER2 gene; thus, these cases should not be interpreted as amplified

a reference laboratory. If a laboratory validated for FISH testing introduces a new *in situ* hybridisation technique, such as CISH, the laboratory may internally validate the technique, by comparing the new technique with FISH. A concordance level between both techniques of at least 95% should be obtained.

The use of standardised kits requires the strict fulfilment of the manufacturer's instructions, without introducing any change. In order to appropriately diagnose polysomic breast carcinomas, it is highly recommended to use kits including centromeric probes. The optimal number of annual cases reported to guarantee the laboratory's technical sufficiency is 100 tests.

Controls

In HIS techniques, the laboratory's own study case serves as an internal control, since it always shows signals both in tumour cells and in normal associated cells (lymphocytes, fibroblasts, normal breast cells, etc). However, if lack of hybridisation is due to technical problems or to the sample, the use of an external control with the same laboratory's fixation and processing conditions will be helpful for the interpretation of results.

Exclusion criteria to perform or interpret an HER2 IHS assay

A test will be considered inappropriate to assess the number of HER2 gene copies when the mentioned pre-analytical (fixation) and analytical (microtomy, immunostaining) minimum requirements are not attained. In addition, a test will be considered inappropriate in the absence of an infiltrative component of the tumour in the slide or presence of a microinfiltrative carcinoma that would be difficult to identify in the fluorescence microscope evaluation (<20 cells), and when a FISH test is carried out (Table 3).

Interpretation criteria

A pathologist should evaluate the results. If another professional is responsible for the interpretation of results, a pathologist must confirm that the result is correct and that invasive tumour was evaluated.

The analysis should exclusively be based on the infiltrative component of the tumour and should include evaluation of at least 20 tumour cells in at least two different tumour areas. If, due to difficulties of dark field assessment, a FISH technique is used, the following recommendations should be followed:

a. Before testing, a pathologist will select an area of infiltrative carcinoma of at least 1 cm^2 in an H&E slide, avoiding the inclusion of carcinoma *in situ* or necrosis areas. A diamond pen or a permanent marker will be used to mark the backside of the FISH slide selected for hybridisation.

b. Before and during technique visualisation, the H&E slide will be reviewed in order to be familiarised with the preparation, and to guarantee that the fluorescence is performed in the infiltrative carcinoma and not in the *in situ* carcinoma or non-malignant cells (normal ducts, lymphocytes, etc.).

c. Strict adherence to these instructions will avoid the main cause of false-positive and false-negative cases of the FISH technique.

When a double probe is used (double-colour FISH or CISH), the HIS technique will be evaluated according to the following criteria (Table 6):

1. A result will be considered *non-amplified* when the HIS ratio (HER2 gene signals/17 chromosome signals) is <1.8.
2. A result will be considered *amplified* when the HIS ratio (HER2 gene signals/17 chromosome signals) is >2.2.
3. The *equivocal* range for HIS analysis is defined as (HER2 gene signals/17 chromosome signals) ratios from 1.8 to 2.2. In this case, the following recommendations apply (Table 7):

Table 7 Interpretation criteria for *in situ* hybridisation HER2 testing using a double probe in equivocal cases

Count additional nuclei (*n*=40 or 60)
Alternatively, have additional person recount
If HER2/chromosome 17 ratio is ≥2 after reevaluation, the case will be reported as *equivocal amplified*
If HER2/ chromosome17 ratio is <2 after reevaluation, the case will be reported as *equivocal non-amplified*
If the case is still *equivocal* after reevaluation, repeat the test, preferably using a different slide or the surgical specimen if the HIS test has been performed on a biopsy sample

a. Evaluation of a greater number of nuclei (*n*=40 or 60) or evaluation of the same number of cells by a second observer. If the new ratio is ≥2, the case will be reported as equivocal amplified. If the ratio is under 2, the case will be reported as non-amplified equivocal.

b. This procedure will solve most equivocal cases. However, if uncertainty persists, repeating the technique is advisable, preferably (a) on a different slide or (b) on the surgical specimen if HIS testing has been performed on a biopsy [34].

4. *Polysomy* 17 is considered when the number of centromere 17 signals per nucleus is ≥3 [7, 8] and *monosomy* when the number of signals is <1.5 [35]. Monosomy 17 may result in false-positive results when using a double probe. Thus, in some cases of monosomy 17 there is evidence that suggests that the HER2/CEP17 ratio ≥2 is caused by the existence of a unique copy of the centromere 17 and two copies of the HER2 gene; thus, these cases should not be interpreted as amplified [36].

5. A result will be considered *not interpretable* in the presence of at least one of the following circumstances:

a. absence of signals of either probe in at least 20 cells;
b. weak or absent signals in >25% of cells;
c. the evaluation of at least two different areas of infiltrative carcinoma is not possible; or
d. controls do not show the expected result.

The following criteria will be applicable for evaluation of results when HIS fluorescence techniques with only a HER2 gene probe are used:

1. A result will be considered *non-amplified* when <4 signals are observed.

2. A result will be considered *amplified* when >6 signals are observed.

3. A result will be considered *equivocal* when 4–6 signals are observed. In these cases it is recommended to count additional tumour cells or, similarly to HIS with double probe, consider repeating the technique: (a) on a different slide or (b) on the surgical specimen if HIS testing has been performed on a biopsy.

The following criteria will be applicable for interpretation of results when CISH with only a HER2 gene probe is used [37]:

1. A result will be considered *amplified* when >10 hybridisation signals or large signal aggregates are observed in >50% of problem cells.

2. A result will be considered *non-amplified* when 1–5 hybridisation signals are observed in >50% of cells (3–5 signals often correspond to chromosome 17 polysomy, although confirmation is not recommended).

3. If 6–10 signals of hybridisation or discrete signal aggregates are observed in >50% of cells, a CEP17 probe will be tested; and this will be considered amplified when the average CEP17/nucleus signals is 2 (disomy) and *non-amplified polysomy* when the average CEP17/nucleus signals is ≥3. In this third group, the possibility of conducting HIS fluorescence or CISH with the double-probe technique may also be considered.

Staff

In order to guarantee expertise, the number of laboratory technicians conducting the test, as well as pathologists interpreting the test, should be as low as possible. Both technicians and pathologists must undergo a training period. They should periodically validate their training in specific working sessions.

Report

The final report can be adapted to the information system used in each hospital. The specific list of elements recommended to be included in the final report is provided in Table 8. The inclusion of a note in the report indicating, when appropriate, that the laboratory participates in external quality assurance programs (SEAP, UK NEQAS, NordiQC, etc.), or the laboratory has been certified or approved for the procedures relative to HER2 testing is also recommended.

Algorithm for HER2 testing

The algorithm described in Fig. 3 is proposed as the most accepted by the scientific community. In cases of uncertainty between 1+ and 2+, or between 2+ and 3+, the practice of *in situ* hybridisation is recommended. As discussed below, *in situ* hybridisation is also advisable if tissue processing has been suboptimal, or if only a cytologic sample is available. Finally, the possibility of directly performing an *in situ* hybridisation test in the initial evaluation of the tumour is also admitted.

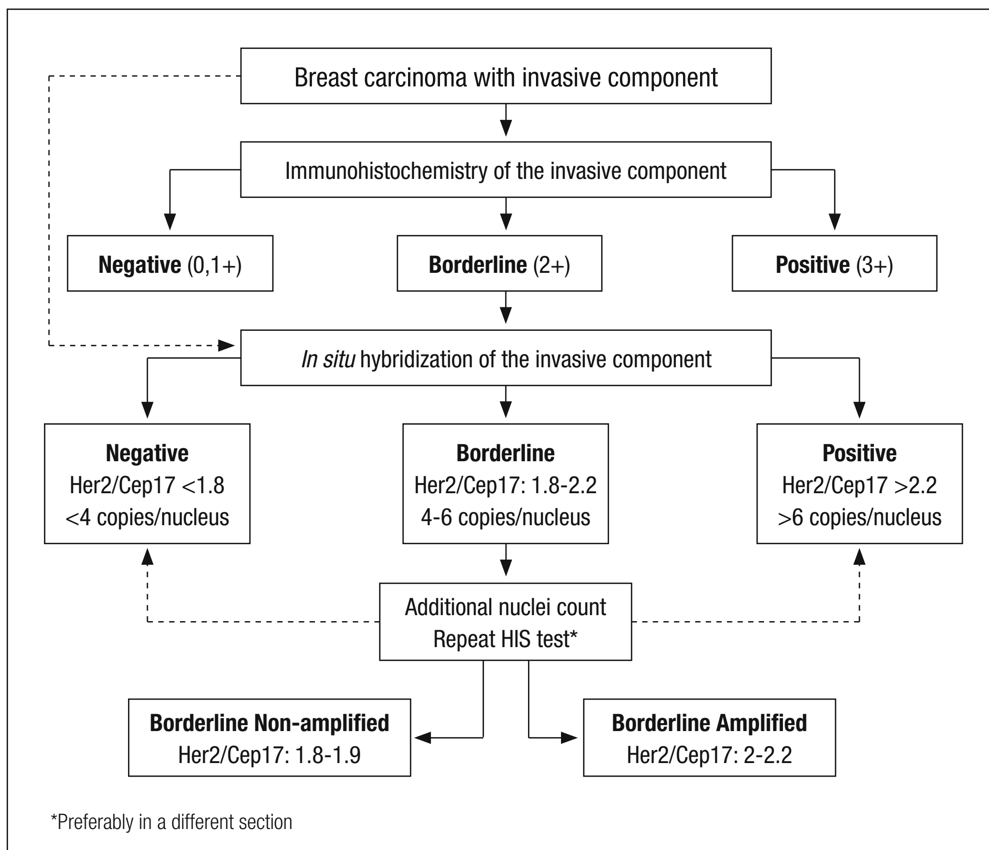

Fig. 3 Algorithm for HER2 testing in breast cancer

Internal quality control

Adequate HER2 testing requires the fulfilment by laboratories of strict quality measures. Internal quality control should include aspects such as procedure standardisation (including the management of samples and the specific HER2 testing method). Thus, documentation in Standardised Operative Procedures (SOPs) is recommended for laboratory structure, organisation, functioning and procedures. Furthermore, an appropriate internal quality control requires the validation of the HER2 testing method and documentation on the validation procedures used when the testing method is modified is particularly relevant. Other elements to consider are the right choice and interpretation

Table 8 Reporting elements for *in situ* hybridisation HER2 testing

Patient identification information
Physician identification
Date of service
Specimen identification (case and block number)
Type of sample and anatomic origin
Fixative type (compulsory), time to fixation (recommendable) and duration of fixation (recommendable)
Probe, specify whether it is approved by the FDA
Number of assessed nuclei
Adequacy of sample for evaluation (appropriate/inappropriate for diagnosis)
Results:
Gene HER2 signals/17 chromosome signals ratio
Presence or absence of polysomy or monosomy
Interpretation of results:
Amplified
Equivocal
Not amplified
Not interpretable

Table 9 Quality criteria for HER2 testing in breast cancer

Appropriate human resources and infrastructure
Standardisation of procedures (standard operative procedures for the management of samples and practice of specific methods)
Use of diagnostic kits approved by regulatory agencies with no changes of the recommended protocol
Initial validation of the testing method: 25 positive and negative cases, checking of the results against a reference laboratory with a level of concordance of 95%
Validation after any change of the testing method
Recommended minimal annual number of tests to guarantee the technical sufficiency:
Immunohistochemistry: 250 tests
In situ hybridisation: 100 tests
Use of the appropriate controls in each testing round
Initial education and regular training of technical and facultative staff
Participation, at least biannually, in external quality assurance programmes (SEAP, UK NEQAS, NordiQC) with optimal results in 90% of the evaluated tests
It is recommended that laboratories initiate the processes for obtaining certification and accreditation of their activities

of controls, and the education and training of the technical staff involved in the practice and interpretation of test, as previously discussed (Table 9).

External quality control

Based on the previously mentioned data from the SEAP Quality Assurance Program, we consider it necessary and convenient that centres using IHC and/or FISH/CISH techniques to evaluate HER2 protein overexpression and gene amplification participate in external quality assurance programs to control the pre-analytical and analytical phases. This is of particular relevance considering the influence of HER2 testing in the treatment options that may be offered to patients.

All consensus publications on HER2 testing [7, 8, 25, 27] underline the need, or the requirement in some cases (UK and Canada), to participate in external quality assurance programmes to retain the laboratory's accreditation to conduct these techniques. The present consensus guidelines recommend the participation in quality assurance programmes at 2-year intervals, considering the test results satisfactory when 90% of the results are optimal. The UK NEQAS (www.ukneqas.org.uk) and the NordiQC (www.nordiqc.org) are internationally well known quality assurance programmes, while the SEAP (www.seap.es) quality assurance programme is well known in our country.

Conflict of interest The authors declare that they have no conflict of interest relating to the publication of this manuscript.

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