Belgian guidelines for HER2/neu testing in breast cancer

Authors C. Colpaert and R. Salgado (on behalf of the Belgian Working Party for Molecular Pathology)

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Summary

The Belgian guidelines for HER2/neu testing in breast cancer are based on the recommendations by the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP), recently published in the Archives of Pathology and Laboratory Medicine[1] and in the Journal of Clinical Oncology.[2] A Review of National Testing Guidelines published in Modern Pathology in 2003 was also taken into account.[3]

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Introduction

Approximately 10-25% of patients with breast cancer have tumours that overexpress human epidermal growth factor receptor 2 (HER2). HER2 positivity is associated with aggressive tumour growth and poor prognosis, but also with response to anti-HER2-targeted therapy. With the expansion of this therapy from the metastatic to the adjuvant setting, accurate assessment of HER2 status has never been more important to ensure identification of all patients who may benefit from anti-HER2-targeted therapy. Moreover, knowledge of HER2 status is also essential for the selection of adjuvant chemotherapy and hormonal therapy. Therefore, accurate HER2 testing of all breast cancer patients at the time of diagnosis is necessary for optimal disease management.

Assay method

Currently, no single assay is globally accepted as the 'gold standard' for HER2 testing. Immunohistochemistry (IHC) is the most often used primary test to detect overexpression of the HER2 protein on the cancer cell membrane. In order to be clinically relevant, the scoring of HER2 results assessed by IHC needs to be semiquantitative rather than qualitative, because a low-level HER2 expression is present in all breast epithelial cells. A relationship between the number of HER2 receptors on a cell's surface and the distribution and intensity of the immunostain was established and the expression of the receptor protein is now routinely graded on a scale from 0 to 3+ (*Table 1*).

For almost all breast cancers, the molecular mechanism underlying HER2 overexpression is amplification of the HER2 gene on chromosome 17. This can be detected with DNA-hybridisation techniques such as Fluorescence-In-Situ-Hybridisation (FISH) or Chromogenic-In-Situ-Hybridisation (CISH). The major advantage of hybridisation techniques is the robustness of the DNA target and the quantitative interpretation of the results, yielding high inter-observer concordance rates.

In single-colour FISH assays, using a DNA probe directed against the HER2 gene, the HER2 status is positive when more than 6 hybridisation signals are present per nucleus.

In dual-colour FISH assays, HER2 probes are used to enumerate HER2 gene copy number, and a second probe - for the centromeric region of chromosome 17 (CEP17) - is used to enumerate chromosome 17 copy number (*Figure 1*). HER2 amplification is observed when HER2/CEP17 ratio ≥2. However, the instructions for the most often used dual-colour

Table 1. Scoring of HER2 immunohistochemistry.
• **Score 0:** no staining is observed in invasive tumour cells ("negative").
• **Score 1+:** weak, incomplete membrane staining in any proportion of invasive tumour cells, or weak, complete membrane staining in less than 10% of cells. Incomplete means that the cells are only stained in part of their membrane ("negative").
• **Score 2+:** complete membrane staining that is non-uniform or weak but with obvious circumferential distribution in at least 10% of invasive tumour cells, or intense complete membrane staining in 30% or less of invasive tumour cells ("equivocal: weakly or focally positive").
• **Score 3+:** intense complete membrane staining is observed in more than 30% of invasive tumour cells ("positive").
Intense staining: easily visualised with 4x or 10x objective *Weak staining: visualisation requires 40x objective*

FISH assays mention that results at or near the cut-off point should be interpreted with caution. In the event of a borderline result (1.8-2.2), particularly if there also appears to be count variability from nucleus to nucleus, 20 additional nuclei should be enumerated. The specimen slide should be re-enumerated by a second person to verify the results. If doubts persist, the assay should be repeated with a fresh specimen slide.

Guideline recommendations by ASCO/CAP

Worldwide, IHC and FISH are the two HER2 assays most commonly used in clinical diagnostics. Patients with strong HER2 overexpression (IHC score 3+) or HER2 gene amplification benefit most from anti-HER2-targeted therapy. National guidelines have taken this into consideration when proposing testing algorithms.

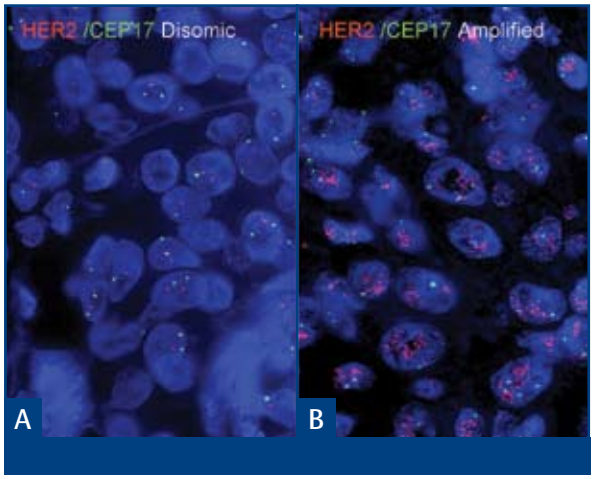

Figure 1. Dual-colour FISH test showing invasive breast carcinoma cells without HER2 gene amplification (a) and invasive breast carcinoma cells with HER2 gene amplification (b). HER2 signals red, chromosome 17 signals green.

In the recommendations given by the American Society of Clinical Oncology and the College of American Pathologists[1,2], a ***positive HER2 test*** is defined as either an IHC result of 3+ cell surface protein expression (defined as uniform intense membrane staining of >30% of invasive tumour cells) or FISH results of amplified HER2 status: >6 HER2 gene copies per nucleus in single-colour FISH assays and HER2/CEP17 ratio > 2.2 in dual-colour FISH assays. The IHC cut-off of more than 30% (rather than the originally specified 10%) reflects the cumulative experience that a high percentage of cells will usually be positive in truly HER2 over-expressing tumours and aims to decrease the incidence of a false positive IHC 3+ score. A high rate of false positives is encountered when the results of centralised HER2-testing, as performed by laboratories involved in multi-centre trials, are compared with the results of laboratories that do not have the quality standards used by centralised laboratories.

A ***negative HER2 test*** is defined as IHC score 0 or 1+ (no staining or weak, complete membrane staining in less than 10% of tumour cells or weak, incomplete membrane staining in any proportion of tumour cells) or a FISH result negative for HER2 gene amplification: HER2 gene copy number <4 per nucleus in the single-colour FISH assays and HER2/CEP17 ratio <1.8 in the dual-colour FISH assays.

An ***equivocal HER2 test*** is defined as IHC score 2+: complete membrane staining that is either non-uniform or weak in intensity but with obvious circumferential distribution in at least 10% of cells. Very rarely, invasive tumours can show intense, complete

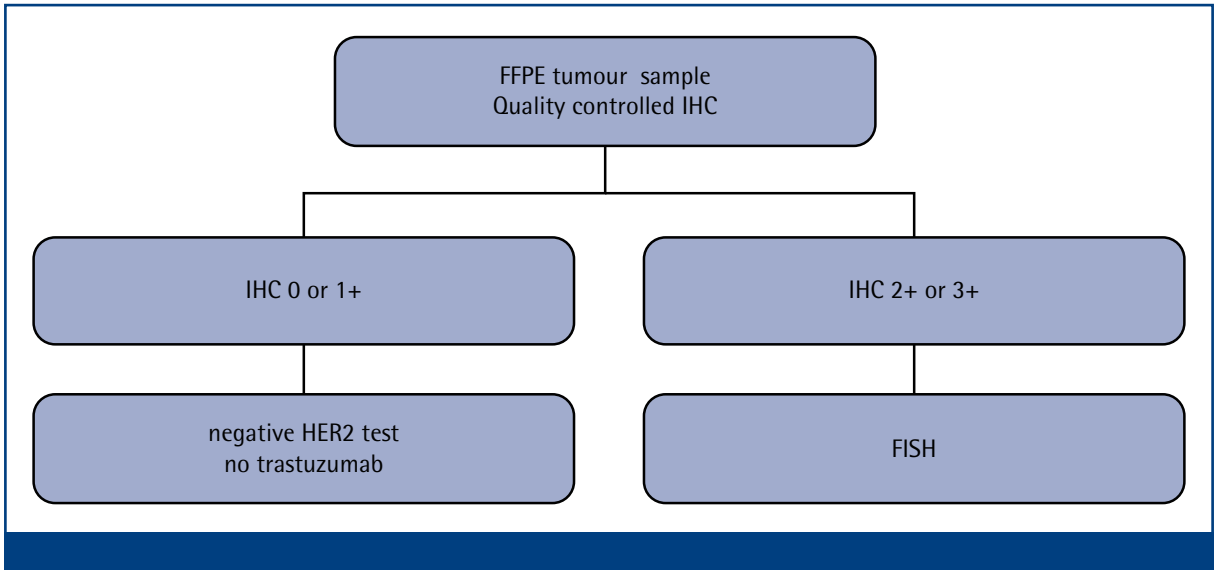

Figure 2. In Belgium, immunohistochemistry (IHC) is used as the primary test to detect overexpression of the HER2 protein. Reflex testing by Fluorescence In-Situ-Hybridisation (FISH) is performed in all tumours with IHC scores 2+ or 3+ . FFPE: formalin fixed paraffin embedded.

membrane staining of 30% or fewer tumour cells. These test results are also considered to be equivocal in the American guideline. The equivocal range for FISH assays is defined as average HER2 gene copy number between 4 and 6 per nucleus for single-colour FISH assays and HER2/CEP17 ratios between 1.8 and 2.2 for dual-colour FISH assays. Patients with equivocal HER2 test results constitute a small and poorly studied subgroup with an uncertain association of test scores and benefit from HER2-directed therapy. By defining the test results as equivocal, further studies of this subgroup will be promoted. However, patients with HER2/CEP17 ratio ≥2 or IHC score 3+ were eligible for treatment in the adjuvant trastuzumab trials. Therefore, available efficacy data do not support excluding them from therapy with trastuzumab at this time.

Belgian reimbursement criteria and testing algorithm

In May 2007, Belgian reimbursement criteria for the use of trastuzumab (Herceptin®) in the adjuvant treatment of primary breast cancer were published in the Moniteur Belge/Belgisch Staatsblad[6], largely based on the American guidelines described above. However, the treatment is only reimbursed if amplification of the HER2 gene has been proven by FISH. Therefore, although immunohistochemistry is used for primary HER2 testing, reflex FISH testing is performed in all tumours with IHC 2+ or 3+ scores (*Figure 2*). An amplified HER2 status is defined as more than 6 HER2 gene copies per nucleus in single-colour FISH assays and HER2/CEP17 ratio > 2.2 in dual-colour FISH assays (*Figure 3 and Figure 4*).

A non-amplified HER2 status is defined as HER2 gene copy number <4 per nucleus in the single-colour FISH assays and HER2/CEP17 ratio <1.8 in the dual-colour FISH assays.

In case of an equivocal test result (average HER2 gene copy number between 4 and 6 per nucleus for single-colour FISH assays and HER2/CEP17 ratios between 1.8 and 2.2 for dual-colour FISH assays), additional tests are needed. First, 20 additional cells will be counted in the FISH assay and the assay will be recounted by a second person. If the result is still equivocal, the FISH test should be repeated and a validated IHC test should be performed by the centre offering the FISH test. The decision to treat the patient with trastuzumab is then based on the results of both FISH tests and the validated IHC test, keeping in mind that patients with HER2/CEP17 ratio ≥2 or IHC score 3+ were eligible for treatment in the adjuvant trastuzumab trials. So far, only one randomised trial selected patients for trastuzumab treatment based solely on HER2 gene copy number visualised by CISH, with a positive cut-off of ≥6 HER2 signals in more than 50% of cells. In equivocal cases, CEP17 hybridisation was added on an adjacent tissue section.

The proposed algorithms may require adjustment as

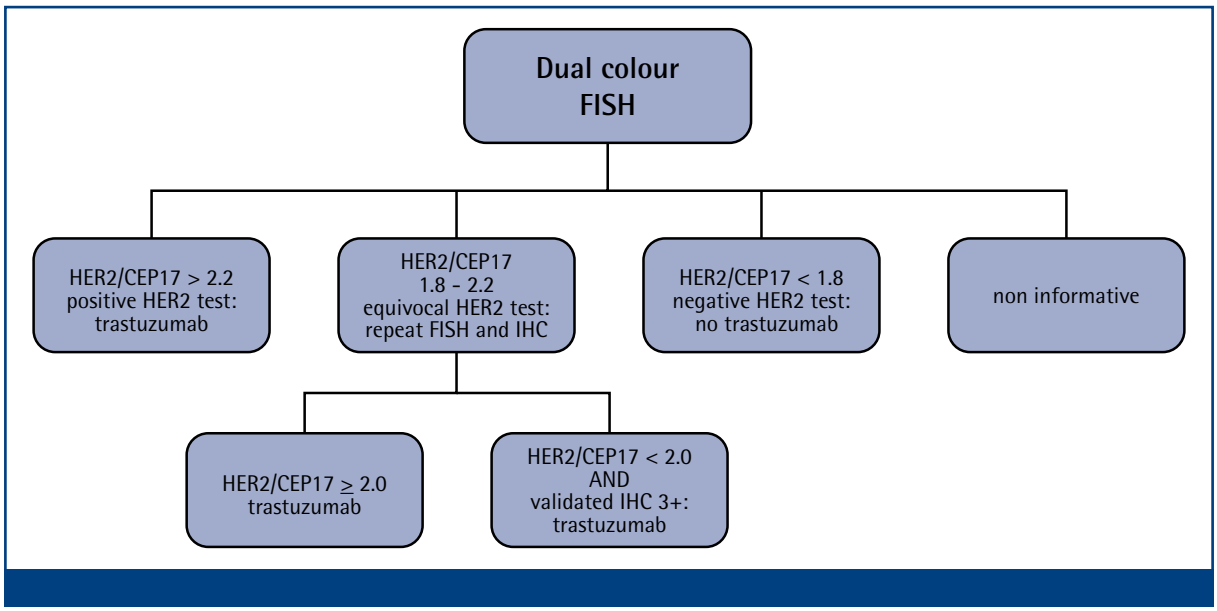

Figure 3. HER2 testing algorithm in Belgium, based on the ASCO/CAP guidelines, when using a dual-colour FISH assay.

more data become available on the efficacy of trastuzumab in the subgroup of patients with equivocal test results.

It should be emphasized that the FISH assay can be non-informative in some cases. Poor tissue fixation may account for FISH assay failure rates up to 10%. The FISH assay should then be repeated on another tissue block from the tumour with better fixation, if available.

Strategies to ensure optimal performance, interpretation and reporting of IHC and FISH assays

Since both IHC and FISH assays are subject to inter-laboratory variation and errors in the preanalytic, analytic and postanalytic phase, quality-control procedures are crucial. The assays should be standardised using written procedures and regularly validated, both internally and externally, using the quality control

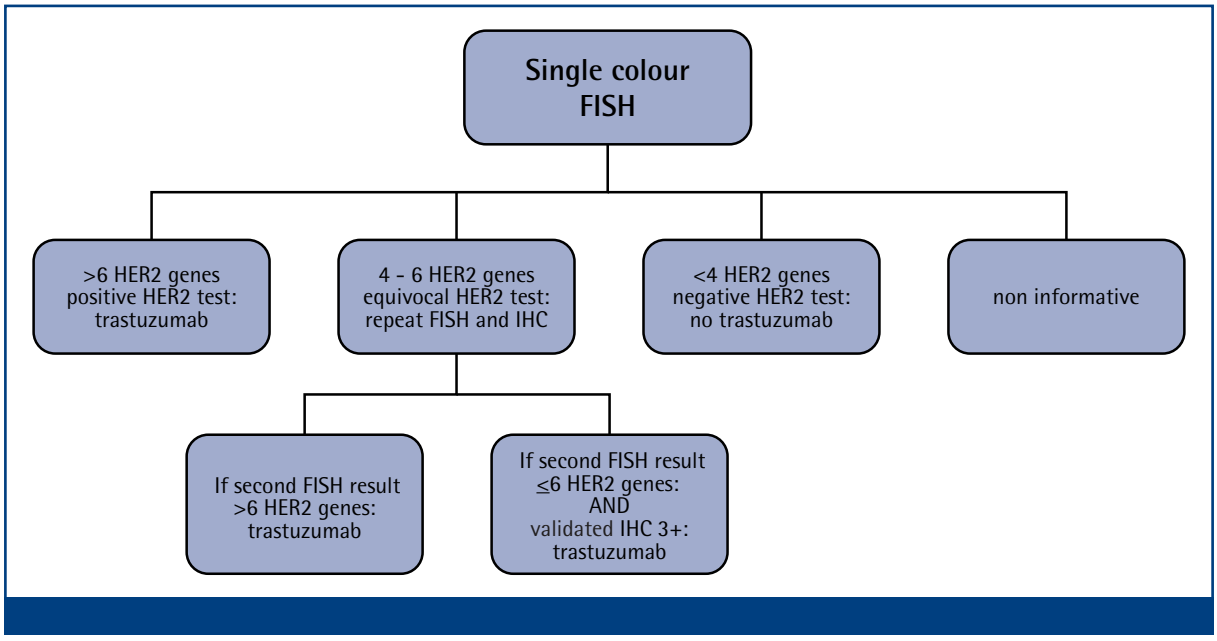

Figure 4. HER2 testing algorithm in Belgium, based on the ASCO/CAP guidelines, when using a single-colour FISH assay.

Table 2. Reporting elements for immunohistochemistry (IHC).

- Patient identification
- Physician identification
- Date of testing
- Specimen identification: case and block number
- Specimen site and type
- Specimen fixative type
- Time to fixation (if available)
- Duration of fixation (if available)
- Antibody clone and vendor
- Method used
- Image analysis method (if used)

Controls:
- high protein over-expression IHC score 3+,
- low-level protein over-expression IHC score 2+,
- no protein over-expression IHC score 0,
- internal control: no protein over-expression in normal ducts or lobules

Adequacy of sample for evaluation

Results:
- percentage of invasive tumour cells exhibiting complete or circumferential membrane staining
- uniformity of staining: present / absent
- intensity of staining: weak / moderate / strong

Interpretation:
- positive: IHC score 3+
- equivocal: IHC score 2+
- negative: IHC score 1+ or 0
- not interpretable

and quality assurance measures described below. The assays should only be evaluated in the invasive component of a breast cancer and not in the in situ component. Experience with the morphology of breast cancer is required, especially in FISH assays, in order to reliably identify invasive cancer cells.

Interpretation criteria for all types of HER2 tests must be standardised and refined, based on interpretation criteria from recent clinical trials and international experience.

Reporting elements for HER2 testing must be standardised (*Table 2 and 3*).

1. Optimal tissue handling requirements

The interval between tissue acquisition and fixation should be as short as possible, preferably less than 1 hour. A joint effort by surgeons and pathologists is needed in order to achieve this goal. After appropriate gross inspection and designation of margins, incisional and excisional breast biopsies should be promptly sliced at 5- to 10- mm intervals and fixed in 10% neutral buffered formalin for intervals ranging from at least 6 hours to no more than 48 hours. Fixation times for needle biopsies have not been specified in the American Guidelines, although they recommend that needle biopsies fixed less than 1 hour in neutral buffered formalin be excluded from HER2 IHC testing. The minimum formalin fixation time for reliable oestrogen receptor IHC testing is 6 hours.[4] Therefore, we recommend a fixation time of between 6 and 48 hours, regardless of the type and size of the specimen. Time of fixation should be routinely recorded for troubleshooting purposes.

2. Optimal IHC testing requirements

Specificity and sensitivity of individual monoclonal and polyclonal anti-HER2 antibodies used in IHC testing differ.[5] Antigen retrieval techniques increase sensitivity at the expense of specificity. If excess antigen retrieval has occurred, normal epithelium will show positive HER2 immunostaining. Tests with strong membrane staining of internal normal ducts or lobules should be rejected. Be aware of retraction artefacts, which may be falsely interpreted as positive. The test should be recalibrated every time new reagents are used or if changes in tissue handling and processing have occurred. The use of controls with known HER2 levels alongside the assay procedure is mandatory. Positive and negative controls, determined by IHC and FISH, are a minimum requirement. An additional control close to cut-off values is also recommended, e.g. a tumour with IHC score 2+ and HER2/CEP17

Table 3. Reporting elements for Fluorescence In Situ Hybridisation (FISH).	
• Patient identification • Physician identification • Date of testing • Specimen identification: case and block number • Specimen site and type • Specimen fixative type • Time to fixation (if available) • Duration of fixation (if available) • Probes identification • Method used • Image analysis method (if used)	
Controls:	- amplified - equivocal - non-amplified - internal: normal chromosome 17 and HER2 gene copy number in normal ducts or lobules
Adequacy of sample for evaluation: adequate number of invasive tumour cells present	
Results:	- number of invasive tumour cells counted - number of observers - average number of HER2 signals per nucleus or tile (tile is the unit used in image analysis systems) - average number of CEP17 chromosome probes per nucleus or tile - ratio of average HER2 signals / CEP17 signals
Interpretation:	- amplified - equivocal - not amplified - not interpretable

between 2.0 and 2.5. Adequate control materials include cell lines or tumour blocks with well defined negative, equivocal and positive protein expression and gene amplification assays. Control materials should preferably be placed on the same slide as the patient's tumour to prevent technical errors, e.g. failure to apply reagents.
Sections should not be used for HER2 testing if cut more than 6 weeks earlier.

3. Optimal FISH testing requirements

Tissues should be fixed in 10% buffered formalin for 6-48 hours. Other fixatives can lead to DNA degradation or high background autofluorescence. Inadequate fixation may also contribute to background autofluorescence. Overfixation results in extensive protein cross-linking and will require more aggressive tissue permeabilisation to achieve DNA hybridisation, which could lead to loss of signal and poor morphological preservation. Interpretation must include counting of at least 20 nonoverlapping cells in two separate areas of invasive cancer. Counting can be done by a trained technologist, but a pathologist must confirm that the result is correct and that invasive tumour was counted. The test is rejected and repeated if controls are not as expected, >25% of signals are unscorable due to weak signals, >10% of signals occur over cytoplasm, nuclear resolution is poor or autofluorescence is strong.

4. Optimal internal validation procedures (quality control)

The term *quality control* describes the internal validation procedures needed to guarantee the accuracy of test results.
Standard operating procedures must be developed for the IHC or FISH test to be offered.
Validation of the test must be performed before the test is offered. This means that testing must occur in parallel with an alternative, validated method in the same laboratory or with the same validated method in another laboratory. This may be organized through exchange programs with laboratories with validated FISH and/or IHC HER2-protocols. The number of tests required for a reliable validation is not well defined, but ranges from 25 to 100 cases and depends on the variety of possible results and the variation in results encountered in the test.

Key messages for clinical practice

1. Accurate HER2 testing of all breast cancer patients at the time of diagnosis is necessary for optimal disease management.

2. Patients with strong overexpression of the HER2 protein on the cancer cell membrane or with HER2 gene amplification benefit most from anti-HER2-targeted therapy.

3. In Belgium, immunohistochemistry (IHC) is used for primary HER2 testing, detecting overexpression of the HER2 protein. Fluorescence-In-Situ-Hybridisation (FISH) testing is performed in all tumours with IHC scores 2+ or 3+.

4. Quality-control procedures are needed to ensure optimal performance of IHC and FISH tests.

5. Tumour tissues should be fixed in 10% buffered formalin for 6-48 hours. The interval between tissue acquisition and fixation should be as short as possible, preferably less than 1 hour.

6. The use of controls with known HER2 levels alongside the assay procedure is mandatory. Positive and negative controls, determined by IHC and FISH, are a minimum requirement. An additional control close to cut-off values is also recommended.

7. Laboratories need to document their concordance between IHC and FISH annually. Concordance in IHC 0 and IHC 3+ categories should be at least 95%. Importantly, concordance in IHC 1+ category should be documented to be > 95% before limiting FISH testing only to IHC >2+ results.

8. A yearly audit of HER2-positive results in an unselected breast cancer population should show that these are within the reported limits of 10-25%.

For both positive and negative HER2 categories, the new test should demonstrate concordance of at least 95% with the validated assay it is compared to.
After this initial test validation, laboratories must document their concordance between IHC versus FISH annually. Concordance in IHC 0 and IHC 3+ categories should be at least 95%. Importantly, concordance in IHC 1+ category should be documented to be >95% before limiting FISH testing only to IHC > 2+ results.
Laboratory personnel and pathologists should be trained and assessed for competency. Individuals interpreting the tests should be 95% concordant with each other (2nd level proficiency testing). Adherence to interpretation criteria and quality control rounds greatly improve inter-observer reproducibility.
A yearly audit of HER2-positive results in an unselected breast cancer population should show that these are within the reported limits of 10-25%.

The association of HER2 status with certain histological types of tumour can also be used as an internal quality control measure. Classic lobular carcinoma, mucinous and tubular carcinomas are usually HER2-negative. Hormone receptor negative, high grade carcinoma with extensive intraductal component and inflammatory carcinoma are usually HER2-positive, and Paget's disease is almost invariably HER2 positive.

5. Optimal external proficiency assessment (quality assurance, QA)

Quality assurance is the technical evaluation that compares the results from one laboratory with those from other laboratories (3rd level proficiency testing). In the US, all laboratories reporting HER2 testing must participate in a guideline concordant proficiency testing (PT) program specific for each assay method used (IHC, FISH). To be concordant, PT programs must distribute specimens at least

twice per year, including a sufficient number of challenging cases to ensure adequate assessment of laboratory performance. Laboratories with less than 90% correct responses must suspend HER2 testing until performance issues are corrected.

In Belgium, participation in external QA programs is mandatory for laboratories performing FISH, but not for laboratories performing only IHC. The Belgian working group of Molecular Pathology organised an external QA round for HER2 IHC and FISH in 2005, limited to the Centres for Molecular Diagnostics. A larger-scale QA round for HER2 IHC is planned for late 2007 with participation by as many laboratories as possible.

6. Optimal laboratory accreditation

In June 2007, the requirements for reimbursement of HER2 FISH testing by social security were published in the Moniteur Belge/Belgisch Staatsblad.[7] The test can only be prescribed within the framework of multidisciplinary cancer care (oncologisch zorgprogramma/programme de soins oncologiques). Laboratories performing the test should be accredited for HER2-FISH according to the ISO 15189 or equivalent norm. Proof of accreditation should be obtained at the latest by July 2009. In the meantime, laboratories should prove that internal quality control procedures and external quality assessment procedures are performed in accordance to national and international standards. This may be controlled by the Scientific Institute for Public Health (WIV/ISP).

Conclusions

The assessment of the HER2 status in individual breast cancer patients is an example of how the use of a laboratory assay as the sole determinant of therapy selection poses a challenge to pathologists performing the assay and oncologists who must rely on them for clinical decision-making.

The increased efforts to ensure optimal performance and accuracy of the test should become the standard, and set the example for other laboratory assays that determine therapeutic decisions. Reimbursement of the test should be proportional to the clinical importance of the test for the individual breast cancer patient.

References

1. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ et al.: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for Human Epidermal Growth Factor Receptor 2 testing in breast cancer. Arch Pathol Lab Med 2007;131:18-43.

2. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ et al.: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for Human Epidermal Growth Factor Receptor 2 testing in breast cancer. JCO 2007;25:1-28.

3. Bilous M, Dowsett M, Hanna W, Isola J, Lebeau A, Moreno A et al.: Current Perspectives on HER2 testing: a review of national testing guidelines. Mod Pathol 2003;16:173-182.

4. Goldstein NS, Ferkowicz M, Odish E, Mani A, Hastah F.: Minimum formalin fixation time for consistent estrogen receptor immunohistochemical staining of invasive breast carcinoma. Am J Clin Pathol. 2003;120:86-92.

5. Ross JS, Fletcher JA, Bloom KJ, Linette GP, Stec J, Symmans WF et al.: Targeted therapy in breast cancer: the HER-2/neu gene and protein. Mol& Cell Proteom 2004; 3:379–398.

6. See: www.ejustice.just.fgov.be/cgi/welcome.pl; numac 2007022786, publication date 2007-05-21.

7. See: www.ejustice.just.fgov.be/cgi/welcome.pl; numac 2007023020, publication date 2007-06-25 Artikel 33bis.

Members of the Belgian working party for molecular pathology

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Correspondence address

First author:
Ms. C. Colpaert MD, PhD
Department of Pathology
AZ Sint-Augustinus
Oosterveldlaan 24
2610 Wilrijk
E-mail: Cecile.Colpaert@gza.be
Tel: 03/4433922

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