HER-2 status determination in breast carcinomas. A practical approach

T. García-Caballero[1], M.D. Menéndez[2], A. Vázquez-Boquete[1], R. Gallego[1], J. Forteza[3] and M. Fraga[3]

[1]Department of Morphological Sciences, [2]Service of Oncology, and [3]Department of Pathology and
Forensic Sciences, Faculty of Medicine and University Clinical Hospital of Santiago de Compostela, Spain

Summary. Accurate evaluation of HER-2 status is crucial in the selection of breast carcinoma patients for trastuzumab (Herceptin) treatment. Various laboratory methods have been used for this purpose. The aim of the present work was to analyse the results obtained in the routine practice by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) in determination of HER-2 status. Five hundred and three cases of breast invasive ductal carcinoma were selected to analyse the HER-2 overexpression by immunohistochemistry (HercepTest, Dako). HercepTest 2+ equivocal cases (60) were studied by FISH (PathVysion, Vysis) to determine HER-2 gene amplification. HER-2 overexpression determined by Herceptest was shown in 97/503 cases (19%). FISH performed on equivocal cases demonstrated HER-2 amplification in 11/60 tumours (18%). IHC and FISH together showed HER-2 overexpression / gene amplification in 21% of breast invasive carcinomas. Immunohistochemical determination of HER-2 status represents an easy and standardized method that (in contrast to FISH) can be performed in all pathology laboratories without need of any special microscope and enabling to check the morphologic features of the cells analysed. However, in order to assure the reliability of the results, standardization of fixation protocols, automation of the immunohistochemical procedure, and training of pathologists in the interpretation of the results (scoring criteria) should be a priority. Equivocal HercepTest cases must be analysed by FISH preferably in a reference laboratory.

Key words: HER-2, Immunohistochemistry, Fluorescence in situ hybridization, FISH, breast carcinoma.

Offprint request to: Tomás García Caballero, Departamento de Ciencias Morfológicas, Facultad de Medicina, 15782 Santiago de Compostela, España. e-mail: cm-tgc@usc.es

Introduction

HER-2/neu (HER-2) is a proto-oncogene located at chromosome 17 that encodes a 185 kDa Human Epidermal growth factor Receptor protein known as HER-2, also called c-erbB-2 or p185 (Schechter et al., 1984; Popescu et al., 1989). HER-2 protein is a member of the type I family of growth receptors, the erbB family of receptor tyrosine kinases, which also includes the HER-1 (EGFR or c-erbB-1), HER-3 (c-erbB-3) and HER-4 (c-erbB-4). HER-2 protein is thought to be involved in the control of cell growth and development and its expression can be detected in many normal tissues. Overexpression of HER-2 protein, which is usually the result of HER-2 gene amplification, results in oncogenic transformation (Chazin et al., 1992).

Since 1955, a modest decrease (7%) in overall breast cancer mortality has occurred in the European Union (Levi et al., 2003). Despite significant improvements in the early diagnosis and treatment of breast cancer and accompanying incremental gains in 5-year survival, a significant number of women will relapse and ultimately die of metastatic disease. Pioneer studies had shown HER-2 gene amplification in 25-30% of human primary breast cancers (Slamon et al., 1987, 1989). Overexpression of HER-2 protein and/or amplification of the HER-2 gene generally appear to be a bad prognostic factor, with more aggressive disease leading to shortened disease-free survival and overall survival when compared with patients bearing HER-2 negative tumours (Slamon et al., 1989; Ross and Fletcher, 1998; Schmidt et al., 2005). A positive HER-2 status in breast cancer may also predict the likelihood of resistance to some conventional therapies (Stal et al., 1995; Newby et al., 1997), as well as possibly being predictive of an increased chemosensitivity to anthracyclines (Paik et al., 1998; Thor et al., 1998). It is now clear that beyond its prognostic and predictive value HER-2 is a highly promising and specific anticancer target. The humanized Ig G1 monoclonal antibody trastuzumab (Herceptin)

(Genentech Inc., San Francisco, CA, USA), binds with high affinity to the ectodomain of HER-2 receptor, thereby inhibiting proliferation of tumour cells that overexpress the HER-2.

The most commonly used methods for HER-2 evaluation are immunohistochemistry (IHC), which detects protein overexpression, and fluorescence in situ hybridization (FISH), which analyses HER-2 gene amplification. Samples scored as 0 or 1+ by HercepTest were considered negative, cases scored 2+ were considered (until recently) weakly positive, and cases scored 3+ were classified as strongly positive for HER-2 protein overexpression. The level of benefit obtained from trastuzumab therapy correlates with the level of HER-2 overexpression as determined by IHC or presence or absence of HER-2 gene amplification, as determined by FISH. In the pivotal phase II trial of single-agent trastuzumab as second/third-line therapy for metastatic disease, Cobleigh et al. (1999) reported that higher levels of HER-2 overexpression (i.e. 3+ versus 2+) predicted for a higher response rate. Moreover, it was demonstrated that most patients (75%) with 2+ results obtained with the HercepTest did not have HER-2 gene amplification (Lebeau et al., 2002; Perez et al., 2002), and the evidence suggests that only tumours with amplification respond to anti-HER-2 therapy (Lewis et al., 2004). With regard to trastuzumab therapy it was then recommended that all specimens with a 2+ Herceptest score be considered equivocal and analysed by FISH to test HER-2 gene amplification (Lebeau et al., 2002; Perez et al., 2002). The subset of 2+ HER-2 cases that are also FISH positive may benefit from trastuzumab.

In view of the importance that accurate HER-2 status evaluation has on patient treatment, we performed a retrospective study of 503 breast cancer cases in order to analyse the results obtained by IHC and FISH and the advantages/disadvantages of each technique.

Materials and methods

Tumour specimens

A total of 503 cases of invasive breast carcinoma diagnosed at the Departments of Pathology of the following Spanish Hospitals: University Clinical Hospital of Santiago de Compostela (Spain), Arquitecto Marcide (Ferrol), Centro Oncológico (A Coruña), Complejo Hospitalario de Pontevedra, POVISA (Vigo), Fundación Jiménez Díaz (Madrid), Juan Canalejo (A Coruña), Marqués de Valdecilla (Santander), Meixoeiro (Vigo), Provincial de Castellón, San Jaime (Torrevieja) and Xeral-Cíes (Vigo), were selected to determine HER-2/neu overexpression. Samples were fixed in 10% buffered formalin for up to 24 hours and embedded in paraffin routinely. Sections 4 µm thick were mounted on ChemMate capillary gap microscope slides (DakoCytomation, Glostrup, Denmark) and heated in an oven at 60°C for 1 hour.

Immunohistochemistry

The IHC technique was automatically performed using a TechMate 500 plus (DakoCytomation). The HercepTest kit (DakoCytomation) was employed following the recommendations provided by the manufacturer. Briefly, after epitope retrieval in 10 mM sodium citrate buffer (pH 6.0) using water bath for 40 minutes at 95-99°C, the slides were allowed to cool for 20 minutes at room temperature and loaded into the TechMate slide holder. The HER-2 immunostaining protocol includes incubation in: 1) primary A485 polyclonal antibody to HER-2 for 25 minutes; 2) peroxidase-blocking reagent 3x2.5 minutes; 3) visualization reagent (dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins) for 30 minutes; 4) 3,3'-diaminobenzidine chromogen solution 3 x 5 minutes; and 5) hematoxylin counterstain for 1 minute. Immunostaining results were scored as 0, 1+, 2+, or 3+ according to the HercepTest guidelines. Scores of 0 and 1+ were considered as negative, score of 2+ was regarded as equivocal or indeterminate, and score of 3+ was informed as positive (overexpression).

Positive controls included: 1) a slide provided with the kit that contains three pelleted, formalin-fixed, paraffin embedded human breast cancer cell lines with scores of 0 (MDA-231 cell line), 1+ (MDA-175) and 3+ (SK-BR-3) (Fig. 1A-D), and 2) a 2+ control carcinoma fixed and processed in the same manner as the patient samples (in-house tissue control). We used a 2+ scored carcinoma instead of a positive (3+) case in order to detect small changes in sensitivity. Both types of controls were included in each staining run. Sections were observed and photographed using a Provis AX70 microscope (Olympus, Tokyo, Japan).

FISH

The cases scored 2+ (equivocal) by IHC (n = 60) were evaluated by FISH using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, Ill, USA). The kit contains two pre-mixed probes: LSI HER-2 DNA SpectrumOrange probe of 190 kb specific for the gene HER-2 (17q11.2-q12) and CEP 17 DNA SpectrumGreen probe of 5.4 kb specific for the DNA alpha-satellite sequence of chromosome 17 centromere (17p11.1-q11.1). This test allows a simultaneous determination of HER-2 gene copies and chromosome 17 copies. The technique was performed according to the manufacturer's recommendations. Briefly, the sections were pretreated (in 0.2N HCl for 20 min, pretreatment sodium thiocyanate solution (NaSCN) at 80°C in water bath for 30 min, and proteinase K solution at 37°C for 10 min), and fixed (in 10% neutral buffered formalin for 10 min). Denaturation of specimen DNA was performed by immersion in 70% formamide diluted in 2X standard saline citrate buffer (SSC) at 72°C for 5 min. After dehydration with ethanol series, hybridization was

performed in a humidified chamber at 37°C overnight. Post-hybridization washes were done in 2X SSC/0.3% NP-40 at 72°C for 2 min. DAPI nuclear stain and a glass coverslip were applied. Sections were observed and photographed using an Eclipse E400 Nikon fluorescence microscope (Nikon, Tokyo, Japan) equipped with DAPI (nuclei), and Texas red (HER-2) / FITC (CEP 17) dual bandpass filter sets. The criterion for gene amplification was the HER-2 to CEP 17 ratio >2 (Pauletti et al., 2000).

Positive controls included: 1) a slide provided with the HercepTest kit (Fig. 2A,B), or 2) an amplified control carcinoma fixed and processed in the same manner as the patient samples (in-house tissue control). The first control was used in order to adjust the technique and the second one was included in all subsequent staining runs.

Results

Immunohistochemistry

Only membrane immunoreactivity was evaluated. Diffuse or punctuated cytoplasmic staining not accompanied by membrane positivity was considered non-specific. Following Herceptest guidelines, scoring criteria were as follows: score of 0, no staining at all or membrane staining in less than 10% of the tumour cells observed (Fig. 1E); score of 1+, more than 10% of the tumour cells showed a faint/barely staining only in part of their membrane (Fig. 1F); score of 2+, a weak to moderate staining of the entire membrane is observed in more than 10% of the tumour cells (Fig. 1G); score of 3+, a strong staining of the entire membrane is observed in more than 10% of the tumour cells (Fig. 1H). The sections were first evaluated at low power magnification that allowed us to establish an easy determination of scores of 0 and 3+ (Fig. 1I). Higher magnification (x20 and x40 objectives) was used to confirm the membrane immunostaining pattern. More difficult was to distinguish between scores of 1+ and 2+ that usually showed a mix of cells with complete and incomplete membrane staining. In these cases, if the percentage of cells with complete membrane staining was less than 10% the score was 1+. Difficulties between scores of 2+ and 3+ were not frequent, but in borderline cases if more than 80% of cells show intense membrane staining the

score is 3+.

Controls performed confirmed the specificity and the sensitivity of the technique. No immunostaining was found in 0 control cell lines and intense immunoreactivity was observed in 3+ control cell lines of all staining runs. The 1+ control cell line and the 2+ in-house tissue control allowed us to detect a very occasional decrease in the sensitivity of the technique. In these cases, the replacement of the substrate-chromogen solution (DAB) for a new one usually resolved the problem. No immunostaining was shown in normal ductal-lobular epithelial system of the mammary gland with the HercepTest (Fig. 1J).

A total of 503 cases of invasive ductal carcinoma of breast were analysed. The number of cases in each HercepTest immunostaining group was as follows: 282 cases (56%) were scored as 0; 64 cases (13%) as 1+; 60 cases (12%) as 2+; and 97 cases (19%) as 3+. Indeed, 69% of cases were negative (0 and 1+), 12% were equivocal (2+), and 19% were positive (3+) for HER-2 protein overexpression (Table 1).

The results of the oestrogen receptor expression were also analysed. Oestrogen receptor expression was found in 55% of HER-2 positive tumours, a result statistically significantly lower (as determined by chi-square, $p < 0.0001$) than the 82% found in HER-2 negative carcinomas.

FISH

FISH analysis was successfully performed on all 60 cases previously scored 2+ by HercepTest. For evaluation of gene amplification 60 randomly selected nuclei of each case were scored at x100 magnification. Nuclear boundaries were analysed by DAPI labelling and overlapping nuclei were excluded. Cases with an average HER-2 to CEP 17 signal ratio more than 2 were considered to be amplified (Fig. 2C-E). HER-2 amplification was detected in 11 (18%) of 60 cases analysed. Indeed, 108 (21%) of the 503 tumours studied showed HER-2 overexpression / amplification (Table 1).

Discussion

Pathologists now play a more important role in clinical medicine by providing not only microscopic
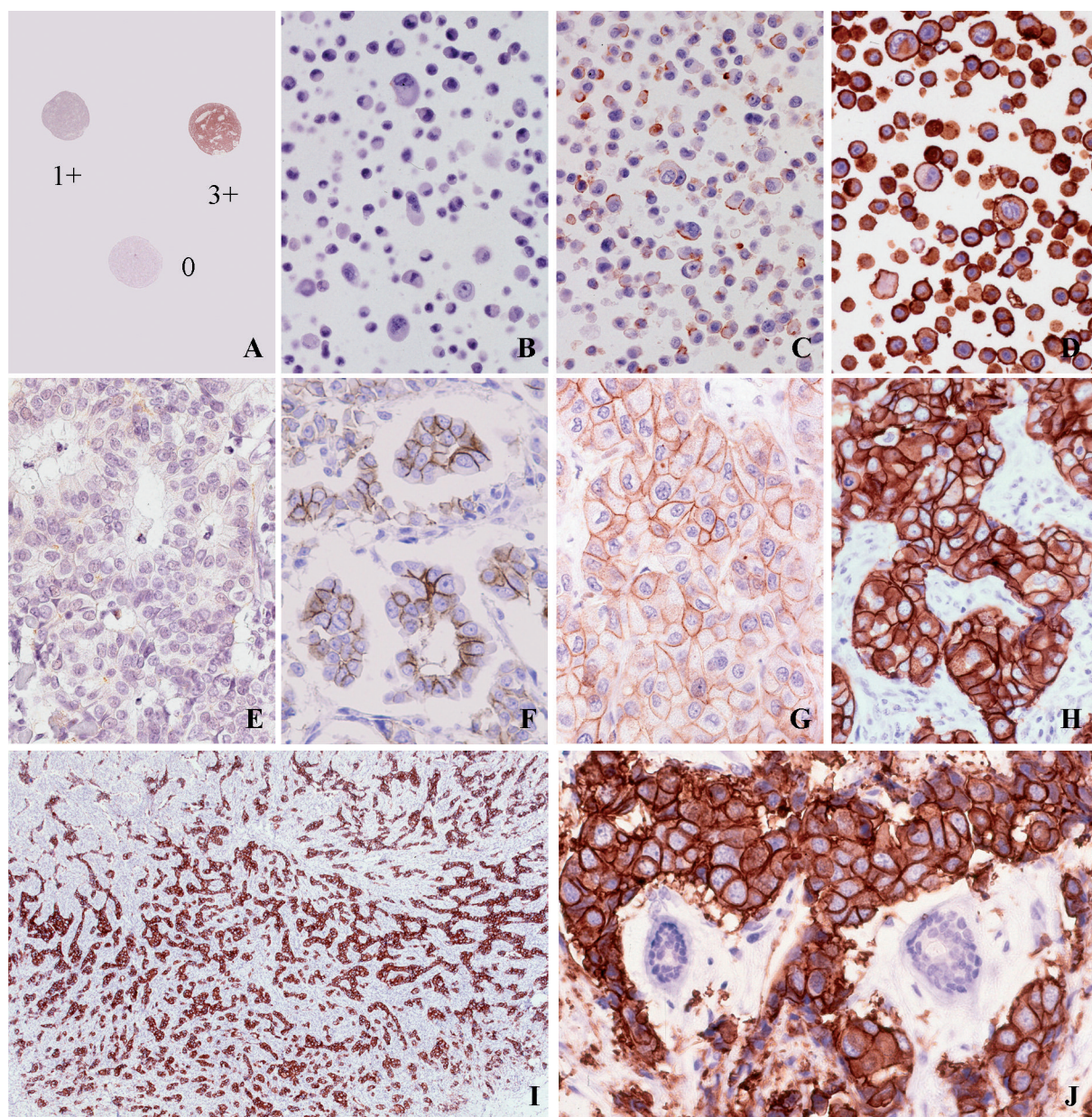
Table 1. Immunohistochemical (HercepTest) and FISH HER-2 results.

IHC (HercepTest)				FISH		Overexpression / Amplification	
Score	No. cases	%	Result	Amplified cases	%	No. cases	%
0	282	56%	Negative				
1+	64	13%	69%				
2+	60	12%	Equivocal 12%	11	18%	11	
3+	97	19%	Positive 19%			97	21%
Total	503			11/60		108/503	

diagnostics, but also laboratory determinations responsible for the selection of patients for targeted therapies (Zarbo and Hammond, 2003). Determination of HER-2 status is useful primarily to select patients with metastatic disease for trastuzumab (Herceptin) therapy, but HER-2 has also a clinical value as a purely prognostic factor and as a predictive factor of a good therapeutic response to anthracyclines and taxanes and a

Fig. 1. Immunohistochemical (HercepTest) HER-2 results. **A.** Control slide that contains 0, 1+ and 3+ cell lines (macrophotography). **B.** Control cell line MDA-231 (0). No immunoreactivity demonstrates the specificity of the test (x 40). **C.** Control cell line MDA-175 (1+). Partial membrane staining present in a small number of cells validates the sensitivity of the assay. These cells also show occasional dot-like immunostaining of the Golgi region (x 40). **D.** Control cell line SK-BR-3 (3+). Complete and strong membrane staining confirms the correct sensitivity of the technique (x 40). **E.** Negative HercepTest (0) tumour. No immunoreactivity was found in this case (x 40). **F.** Negative HercepTest (1+) tumour. Incomplete membrane labelling is shown in more than 10% of cells (x 40). **G.** Equivocal HercepTest (2+) tumour. Complete membrane staining of weak intensity is seen in more than 10% of cells (x 40). **H.** Positive HercepTest (3+) tumour. Complete and intense membrane staining is found in virtually all tumour cells (x 40). **I.** Positive Herceptest (3+) tumour. Intense staining is observed even at low magnification (x 4). **J.** Positive Herceptest (3+) tumour. Complete membrane staining of strong intensity is shown in the tumour cells whereas the normal ducts were negative (x 40).

poor response to hormonal therapy (Stal et al., 1995; Newby et al., 1997; Paik et al., 1998; Thor et al., 1998; Hayes and Thor, 2002). As proposed by the College of American Pathologists (Zarbo and Hammond, 2003) we tested HER-2 in every newly diagnosed patient with invasive breast cancer and not just in those with metastatic disease.

Very recently, Ellis et al. (2005) and Schmidt et al. (2005) reported HER-2 overexpression in 18% and 20% of breast tumours, respectively. These overexpression rates are similar to that obtained in the current study (19%). Our global result (21% of overexpression / gene amplification) agreed exactly with that obtained by Pauletti et al. (2000) by FISH on 900 patients, was similar to the 18.4% obtained by Ridolfi et al. (2000) on

750 cases by IHC and/or FISH, and was higher to that reported by Perez et al. (2002) on 1556 specimens (they found 13% of 3+ cases and amplification in 12% of 2+ specimens). Our percentage of cases scored as 0 is also higher to that reported by these authors (56% vs. 38%), but taking into account all negative cases (0 and 1+) the results were similar (69% vs. 73%).

Bilous et al. (2003) analysed 1536 breast cancers and observed that 95.5% of the 3+ cases were infiltrating ductal NOS and 97% were histological grade 2 or 3. None of "special types" and only 0.8% of infiltrating lobular carcinomas showed overexpression. This result agrees with the finding of HER-2 overexpression in the pleomorphic lobular carcinoma, an aggressive variant of infiltrating lobular carcinoma (Middleton et al., 2000).
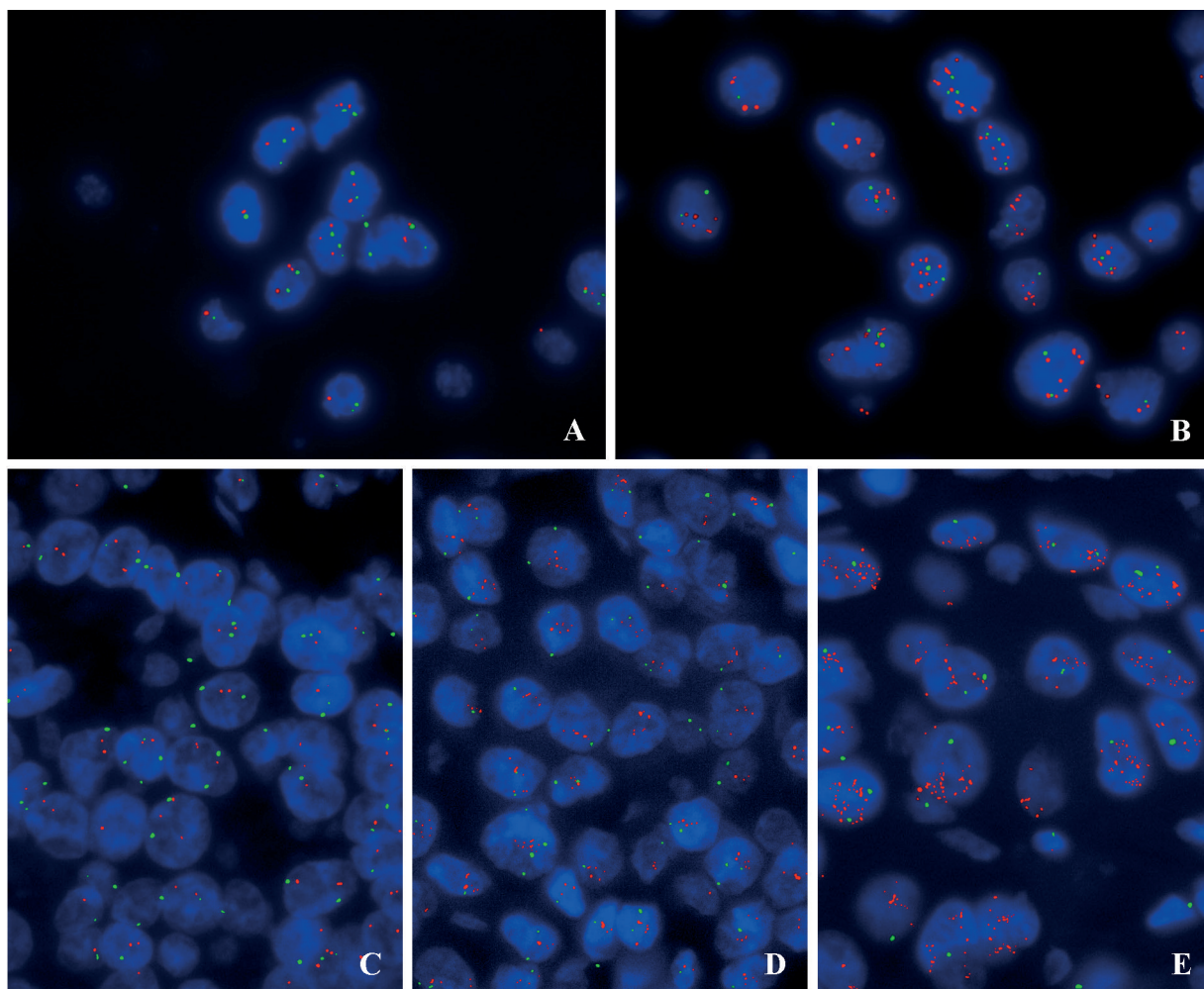
Fig. 2. FISH HER-2 results. Red signals show HER-2 gene and green signals represent centromere of chromosome 17. Captured images from DAPI and Texas red/FITC filters were merged using Adobe Photoshop image treatment software. **A.** Control cell line MDA-231. No HER-2 amplification was confirmed by the similar number of red and green signals (x 100). **B.** Control cell line SK-BR-3. HER-2 amplification showing numerous red signals per nucleus in comparison with the green ones (x 100). **C.** HER-2 non-amplified tumour. Balanced disomy was found (x 100). **D.** HER-2 amplified tumour. In this case the ratio between red and green signals was 2.7 (x 100). **E.** HER-2 amplified tumour. This carcinoma presented a very high amplification with more than 20 red signals per nucleus (x 100).

An inverse association was generally reported between HER-2 overexpression and steroid receptor status (Zeillinger et al., 1989; Marsigliante et al., 1993). Our findings (82% of oestrogen receptor expression in HER-2 negative cases versus 55% in HER-2 positive ones) corroborated this inverse association. Our results also agree with that found by some authors that reported up to 50% of hormone receptor positivity in HER-2 positive cases (Quenel et al., 1995; Andrulis et al., 1998). Moreover, it was recently shown that considering the quantitative levels of HER-2 and hormone receptors as continuous variables, patients with higher levels of HER-2 overexpression/amplification had lower levels of hormone receptors than patients with lower levels of HER-2, a fact that may explain the relative resistance of HER-2- and hormone receptors-positive tumours to hormone therapy (Konecny et al., 2003).

In contrast to the estrogen and progesterone receptors that show positivity in normal ductal-lobular epithelial system (internal positive controls), no immunostaining was found for HER-2 in normal mammary gland. Therefore, it is important to include control slides in each staining run. Quality controls ascertained the validity of the assays. Non-specific background staining was never found in the 0 control cell line and the 3+ control showed an intense immunoreactivity in all staining runs. The most useful control was the 1+ cell line that allowed us to detect a subtle decrease in the sensitivity of the technique. It is desirable that the control slide provided with the HercepTest kit includes a 2+ cell line, e.g., MDA-MB-453 (Andersson et al., 2004), for a better validation of the sensitivity of the assay.

Considerable attention has been given to the standardization of methods employed to evaluate the HER-2 status. Although the HercepTest assay is standardized, the scoring system used to evaluate the results is subjective. HercepTest extreme scores of 0 and 3+ were unequivocal and in fact the highest concordance of IHC and FISH was seen with these scores (Zarbo and Hammond, 2003). Disagreement in Herceptest results was found primarily in cases scored 1+ and 2+ (Perez et al., 2002). In all borderline cases it is recommended to perform FISH to establish the unequivocal HER-2 status. Briefly, HER-2 testing by IHC seems a very good first choice methodology that should be complemented by FISH in inconclusive cases (Ridolfi et al., 2000).

HER-2 gene amplification status was evaluated by calculating the ratio of HER-2 gene/chromosome 17 signals obtained by FISH in 60 randomly selected nuclei of each case. If the ratio was >2 the case was considered amplified (Pauletti et al., 2000). However, in practice most tumours could be easily evaluated on simple inspection and the subsequent quantifications corroborate the first evaluation. Recently a ratio >2.2 was employed for amplified cases and a ratio <1.8 for unamplified cases. Samples scored between 1.8 and 2.2 were considered borderline and must be reenumerated by another qualified observer to ensure the accuracy of the test results (O'Grady et al., 2003; Zarbo and Hammond, 2003; Hicks and Tubbs, 2005). The FISH evaluation requires a fluorescence microscope equipped with an adequate set of filters (not normally available in the majority of pathology laboratories), it must be performed in a dark-room using immersion objectives and it is time consuming. For this reason, alternative reading methods of counting 60 signals instead of 60 nuclei (Olsen et al., 2004) or the signals of only 20 nuclei (DakoCytomation) were proposed with equally good results. The development of automated platforms for hybridization, and image analysis for signal enumeration should broaden the availability of this technology for clinical diagnostic testing (Hicks and Tubbs, 2005).

In cases that present both carcinoma *in situ* and invasive carcinoma, only the invasive component should be scored. For IHC there is no problem, but with the immersion objective in the dark field of FISH the distinction between invasive and *in situ* components is not always easy (Zarbo and Hammond, 2003). In order to avoid this problem, we recommend selecting a block without or with a minimum of *in situ* component for FISH technique. We can also mark on the coverslip of HE the areas that only contain invasive carcinoma and remove the remainder areas with *in situ* component from the new section before performing the FISH assay. In this way, we guarantee HER-2 assessment in invasive areas and we also save probe and time evaluation. Tissue microarray technique can also be used in order to sample precise areas of invasive carcinoma. Alternatively, we can perform FISH on sections of a metastatic lymph node, since HER-2 expression is usually identical in the primary tumour and corresponding metastases (Niehans et al., 1993).

Strict adherence to test protocols and quality control programmes allows a remarkable reproducibility in the results both by IHC (Rhodes et al., 2002) and FISH (Nagle et al., 2002) and a good correlation between both techniques has been established (Hoang et al, 2000; Lehr et al., 2001; Dolan and Snover, 2005; Lottner et al., 2005).

Although IHC and FISH are accurate and precise techniques for HER-2 testing, both methods have a "gray zone", i.e., cases 2+ by IHC and cases with low level of amplification by FISH (Yaziji and Gown, 2004). Lack of concordance between overexpression detected by immunohistochemistry and gene amplification detected by FISH was primarily shown in the past when cases scored 2+ by HercepTest were interpreted as positive (Hoang et al., 2000). In fact, recent reports that consider positive only 3+ cases showed a 98-100% concordance between IHC and FISH (Bilous et al., 2003; Anderson et al., 2003). On the other hand, only rare cases (1-2%) were reported to show gene amplification in absence of protein overexpression (Jacobs et al., 1999; Hoang et al, 2000). In these cases the different effects of fixation on immunohistochemistry and FISH techniques could explain the discrepancies. Overexpression in

absence of amplification was also occasionally observed (Pauletti et al., 2000; Olsen et al., 2004) and it was explained by upregulation or decreased degradation of the protein, although an alternative explanation may be false-positive IHC or false-negative FISH (Olsen et al., 2004).

In order for standards to be appropriately used in testing patients for HER-2 gene and gene protein expression, it was agreed that all tissues for HER-2 testing should be fixed in 10% buffered formalin (Zarbo and Hammond, 2003; Hammond et al., 2003). Length of formalin fixation and delay in fixation may affect the results. Optimal formalin fixation time for HER-2 testing by IHC is 6 to 12 hours (Zarbo and Hammond, 2003). Standardization of fixation procedures and use of automated image analysis may increase the precision of IHC testing (Bartlett et al., 2003). The effect of fixation period on HER-2 gene amplification detected by FISH was studied by Selvarajan et al. (2002). These authors concluded that reliable results for HER-2 amplification were not compromised by the usual range of routine fixation periods of surgical breast specimens. Only a fixation period of more than a week appears to compromise the results. Recently, Hicks and Tubbs (2005) recommended a fixation time of 4-12 hours to optimize FISH assay performance. Poor tissue quality can be easily identified in FISH analyses because of a lack of hybridization signals. Therefore, inappropriate tissue handling is more dangerous in immunohistochemistry because an artificial lack of staining can be regarded as negative results (Tapia et al., 2004).

FISH is not always feasible in routine practice. The introduction of chromogenic *in situ* hybridization (CISH) for detection of HER-2 amplification (Dandachi et al., 2002) avoids the use of fluorescence microscopy and offers the ability to view the morphological features of the cells analysed using traditional brightfield microscopy (Gupta et al., 2003; Hauser-Kronberger and Dandachi, 2004; Wixom et al., 2004). The concordance between CISH and FISH was 96-100% in different series reported (Dandachi et al., 2002; Arnould et al., 2003; Gupta et al., 2003; Hauser-Kronberger and Dandachi, 2004) and CISH was recently approved by the European Commision for assessment of HER-2 status in breast cancer (Penault-Llorca and Cayre, 2004). However, CISH do not allow seeing simultaneously HER-2 and centromere 17, and it was demonstrated that a small number of cases displaying low level amplification by CISH contained chromosome 17 polysomy by FISH (Diaz et al., 2004; Wixom et al., 2004).

Multiparameter DNA flow cytometry (Leers et al., 2003) and quantitative real-time PCR assay (Königshoff et al., 2003) were developed for assessment of HER-2 gene amplification. Concordance rates between real-time PCR and immunohistochemistry and FISH were 91% and 92%, respectively (Schlemmer et al., 2004). However, PCR requires microdissection and in general any technique that does not preserve tissue architecture becomes a less favourable method for evaluation of HER-2 status (Andersson et al., 2004). The validation of tissue microarray technology for detection of HER-2 gene amplification (Diaz et al., 2004) opens the possibility for use of cores in PCR assays, obviating the need for microdissection (Lewis et al., 2004).

The use of genetically modified chimeric/humanized monoclonal antibodies has been first validated by the data from the clinical trials with trastuzumab. Results of the three major fully published clinical trials demonstrated that trastuzumab is active as monotherapy (Cobleigh et al., 1999; Vogel et al., 2002) and has greater antitumour effects than chemotherapy alone (Slamon et al., 2001) when coadministered to patients with metastatic breast cancer overexpressing the HER-2 receptor. In the pivotal randomized controlled single-blind trial reported by Slamon et al. (2001) the addition of trastuzumab to conventional chemotherapy (anthracycline, and cyclophosphamide or paclitaxel) was associated with a significantly longer median time to disease progression, time to treatment failure and duration of response, and a higher rate of overall response compared with chemotherapy alone. In addition, overall survival was improved in the groups which received trastuzumab compared with patients receiving chemotherapy only (25.1 vs. 20.3 months). This result was achieved despite 66% of patients initially randomized to receive chemotherapy alone choosing to receive trastuzumab at disease progression. Trastuzumab-treated groups had a 20% lower relative risk of death at a median follow-up of 30 months (Slamon et al., 2001).

Trastuzumab is generally well tolerated by the patients. The most significant adverse effects being acute fever and/or chills, and the potential to cause cardiac dysfunction (27% of patients receiving an anthracycline and cyclophosphamide combined with trastuzumab, 13% receiving trastuzumab plus paclitaxel and 4.7% receiving trastuzumab alone) (Cobleigh et al., 1999; Slamon et al., 2001).

As stated in the introduction, positive HER-2 status may also be predictive of an increased chemosensitivity to anthracyclines (Paik et al., 1998; Thor et al., 1998). However, more recent studies showed that the response to anthracyclines was obtained only in HER-2 amplified cases that also had amplification of topoisomerase II alpha (TOPIIa) gene (Coon et al, 2002; Di Leo et al., 2002). Amplification and deletion of TOPIIa may account for both relative chemosensitivity and resistance to anthracycline therapy (Järvinen and Liu, 2003), suggesting the need to perform TOPIIa FISH determination in HER-2 amplified cases.

Although trastuzumab has demonstrated synergistic and additive action with several chemotherapy agents preclinically, the optimal combination clinically is yet to be determined. Results of current non-comparative trials of combination therapy may aid in the design of the next generation of randomized comparative studies.

Antineoplastic agents currently being investigated in combination with trastuzumab include paclitaxel, docetaxel, capecitabine, vinorelbine, gemcitabine, carboplatin and cisplatin. Further studies are also investigating the use of trastuzumab in the neoadjuvant and adjuvant setting, in patients with non-metastatic breast cancer (McNeil, 2000; Burstein et al., 2003).

In conclusion, determination of HER-2 has a prognostic and predictive value for the patient and physician, and can be readily performed in most hospitals as part of the routine clinical assessment for every newly diagnosed patient with invasive breast cancer, since the knowledge of HER-2 status is essential not only in stratifying patients to anti-HER-2 therapy regimes, but also as a predictive factor of chemotherapy response. Immunohistochemical determination of HER-2 status represents an easy and standardized method that could be performed in all pathology laboratories without the need of any special microscope and allowing to check the morphologic features of the cells analysed (in contrast to FISH). However, in order to assure the reliability of the results, standardization of fixation protocols, automation of the immunohistochemical procedure, and training of pathologists in the scoring of the results should be a priority. Equivocal HercepTest cases must be analysed by FISH (preferably in a reference laboratory), and in a near future probably by CISH in all pathology departments.

References

Anderson J.M., Ariga R., Govil H., Bloom K.J., Francescatti D., Reddy V.B., Gould V.E. and Gattuso P. (2003). Assessment of HER-2/neu status by immunohistochemistry and fluorescence in situ hybridization in mammary Paget disease and underlying carcinoma. Appl. Immunohistochem. Mol. Morphol. 11, 120-124.

Andersson J., Linderholm B., Bergh J. and Elmberger G. (2004). HER-2neu (c-erbB-2) evaluation in primary breast carcinoma by fluorescent in situ hybridization and immunohistochemistry with special focus on intratumor heterogeneity and comparison of invasive and in situ components. Appl. Immunohistochem. Mol. Morph. 12, 14-20.

Andrulis I.L., Bull S.B., Blackstein M.E., Sutherland D., Mak C., Sidlofsky S., Pritzker K.P., Hartwick R.W., Hanna W., Lickley L., Wilkinson R., Qizilbash A., Ambus U., Lipa M., Weizel H., Katz A., Baida M., Mariz S., Stoik G., Dacamara P., Strongitharm D., Geddie W. and McCready D. (1998). neu/erbB-2 amplification identifies a poor prognosis group of women with node-negative breast cancer.

J. Clin. Oncol. 16, 1340-1349.

Arnould L., Denoux Y., MacGrogan G., Penault-Llorca F., Fiche M., Treilleux I., Mathieu M.C., Vincent-Salomon A., Vilain M.O. and Couturier J. (2003). Agreement between chromogenic in situ hybridization (CISH) and FISH in the determination of HER2 status in breast cancer. Br. J. Cancer 88, 1587-1591.

Bartlett J., Mallon E. and Cooke T. (2003). The clinical evaluation of HER-2 status: which test to use? J. Pathol. 199, 411-417.

Bilous M., Ades C., Armes J., Bishop J., Brown R., Cooke B., Cummings M., Farshid G., Field A., Morey A., McKenzie P., Raymond W., Robbins P. and Tan L. (2003). Predicting the HER2 status of breast cancer from basic histopathology data: an analysis of 1500 breast cancer as part of the HER2000 International Study. Breast 12, 92-98.

Burstein H.J., Harris L.N., Gelman R., Lester S.C., Nunes R.A., Kaelin C.M., Parker L.M., Ellisen L.W., Kuter I., Gadd M.A., Christian R.L., Kennedy P.R., Borges V.F., Bunnell C.A., Younger J., Smith B.L. and Winer E.P. (2003). Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. J. Clin. Oncol. 21, 46-53.

Chazin V.R., Kaleko M., Miller A.D. and Slamon D.J. (1992). Transformation mediated by the human HER-2 gene independent of epidermal growth factor receptor. Oncogene 7, 1859-1866.

Cobleigh M.A., Vogel C.L., Tripathy D., Robert N.J., Scholl S., Fehrenbacher L., Wolter J.M., Paton V., Shak S., Lieberman G. and Slamon D.J. (1999). Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J. Clin. Oncol. 17, 2639-2648.

Coon J.S., Marcus E., Gupta-Burt S., Seelig S., Jacobson K., Chen S., Renta V., Fronda G. and Preisler H.D. (2002). Amplification and overexpression of topoisomerase IIa predict response to anthracycline-based therapy in locally advanced breast cancer. Clin. Cancer Res. 8, 1061-1067.

Dandachi N., Dietze O. and Hauser-Kronberger C. (2002). Chromogenic in situ hybridization: a novel approach to a practical and sensitive method for the detection of HER2 oncogen in archival human breast carcinoma. Lab. Invest. 82, 1007-1014.

Di Leo A., Gancberg D., Larsimont D., Tanner M., Jarvinen T., Rouas G., Dolci S., Leroy J.-Y., Paesmans M., Isola J. and Piccart M.J. (2002). HER-2 amplification and topoisomerase IIa gene aberrations as predictive markers in node-positive breast cancer patients randomly treated either with an anthracycline based therapy or with cyclophosphamide, methothrexate, and 5-fluorouracil. Clin. Cancer Res. 8, 1107-1116.

Diaz L.K., Gupta R., Kidwai N., Sneige N. and Wiley E.L. (2004). The use of TMA for interlaboratory validation of FISH testing for detection of HER2 gene amplification in breast cancer. J. Histochem. Cytochem. 52, 501-507.

Dolan M. and Snover D. (2005). Comparison of immunohistochemical and fluorescence in situ hybridization assessment of HER-2 status in routine practice. Am. J. Clin. Pathol. 123, 766-770.

Ellis C.M., Dyson M.J., Stephenson T.J. and Maltby E.L. (2005). HER2 amplification status in breast cancer: a comparison between immunohistochemical staining and fluorescence in situ hybridization using manual and automated quantitative image analysis scoring techniques. J. Clin. Pathol. 58, 710-714.

Gupta D., Middleton L.P., Whitaker M.J. and Abrams J. (2003). Comparison of fluorescence and chromogenic in situ hybridization for detection of HER-2/neu oncogene in breast cancer. Am. J. Clin. Pathol. 119, 381-387.

Hammond M.E.H., Barker P., Taube S. and Gutman S. (2003). Standard reference material for Her2 testing. Report of a National Institute of Standards and Technology-sponsored Consensus Workshop. Appl. Immunohistochem. Mol. Morphol. 11, 103-106.

Hauser-Kronberger C. and Dandachi N. (2004). Comparison of chromogenic in situ hybridization with other methodologies for HER2 status assessment in breast cancer. J. Mol. Histol. 35, 647-653.

Hayes D.F. and Thor A.D. (2002). c-erb-B-2 in breast cancer: development of a clinically useful marker. Semin. Oncol. 29, 231-245.

Hicks D.G. and Tubbs R.R. (2005). Assessment of the *HER2* status in breast cancer by fluorescence in situ hybridization: a technical review with interpretative guidelines. Hum. Pathol. 36, 250-261.

Hoang M.P., Sahin A.A., Ordoñez N.G. and Sneige N. (2000). HER-2 gene amplification compared with HER-2 protein overexpression and interobserver reproducibility in invasive breast carcinoma. Am. J. Clin. Pathol. 113, 852-859.

Jacobs T.W., Gown A.M., Yaziji H., Barnes M.J. and Schnitt S.J. (1999). Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu breast cancer. J. Clin. Oncol. 17, 1974-1982.

Järvinen T.A.H. and Liu E.T. (2003). *Her-2/neu* and topoisomerase IIa in breast cancer. Breast Cancer Res. Treat. 78, 299-311.

Konecny G., Pauletti G., Pegram M., Untch M., Dandekar S., Aguilar Z., Wilson C., Rong H.-M., Bauerfeind I., Felber M., Wang H.-J., Beryt M., Seshadri R., Hepp H. and Slamon D.J. (2003). Quantitative association between HER-2/neu and steroid hormone receptors in hormone receptor-positive primary breast cancer. J. Natl. Cancer Inst. 95, 142-153.

Königshoff M., Wilhelm J., Bohle R.M., Pingoud A. and Hahn M. (2003). *HER-2/neu* gene copy number quantified by real time PCR: comparison of gene amplification, heterozygosity, and immunohistochemical status in breast cancer tissue. Clin. Chem. 49, 219-229.

Lebeau A., Deimling D., Kaltz C., Sendlehofert A., Iff A., Luthardt B., Untch M. and Löhrs U. (2002). HER-2 analysis in archival tissue samples of human breast cancer: comparison of immunohistochemistry and fluorescence in situ hybridization. J. Clin. Oncol. 19, 354-363.

Leers M.P.G., Hoop J.G.M. and Nap M. (2003). Her2/neu analysis in formalin-fixed, paraffin-embedded breast carcinomas: comparison of immunohistochemistry and multiparameter DNA flow cytometry. Anticancer Res. 23, 999-1006.

Lehr H.-A., Jacobs T.W., Yaziji H., Schnitt S.J. and Gown A.M. (2001). Quantitative evaluation of HER-2 status in breast cancer by fluorescence in situ hybridization and by immunohistochemistry with image analysis. Am. J. Clin. Pathol. 115, 814-822.

Levi F., Lucchini F., Negri E., Boyle P. and La Vecchia C. (2003). Mortality from major cancer sites in the European Union, 1955-1998. Ann. Oncol. 14, 490-495.

Lewis F., Jackson P., Lane S., Coast G. and Hanby A.M. (2004). Testing for HER2 in breast cancer. Histopathology 45, 207-217.

Lottner C., Schwarz S., Diermeier S., Hartmann A., Knuechel R., Hofstaedter F. and Brockhoff G. (2005). Simultaneous detection of HER/neu gene amplification and protein overexpression in paraffin-embedded breast cancer. J. Pathol. 205, 577-584.

Marsigliante S., Muscella A., Ciardo V., Barker S., Leo G., Baker V., Mottaghi A., Vinson G.P. and Storelli C. (1993). Enzyme-linked immunosorbent assay of HER-2/neu gene product (p185) in breast cancer: its correlation with sex steroid receptors, cathepsin D and histologic grades. Cancer Lett. 75, 195-206.

McNeil C. (2000). Herceptin in the adjuvant setting: phase III trials begin. J. Natl. Cancer. Inst. 92, 683-684.

Middleton L.P., Palacios D.M., Bryant B.R., Krebs P., Otis C.N., Merino M.J. (2000). Pleomorphic lobular carcinoma: morphology, immunohistochemistry, and molecular analysis. Am. J. Surg. Pathol. 24, 1650-1656.

Nagle R.B., Tubbs R.R., Roche P.C., Hsi E.D., Linden M.D., Ormsby A., Pettigrew N.M., Rickert R.R., Said J.W., Bayer-Garner I.B., Cooley L.D., Brothman A.R., Persons D.L., Vance G.H., Mascarello J.T., Rao K.W., Herrmann M., Dewald G. and Park J.P. (2002). Clinical laboratory assays for HER-2 amplification and overexpression. Quality assurance, standardization, and proficiency testing. Arch. Pathol. Lab. Med. 126, 803-808.

Newby J.C., Johnston S.R.D., Smith I.E. and Dowsett M. (1997). Expression of epidermal growth factor receptor and c-erb B2 during the development of tamoxifen resistance in human breast cancer. Clin. Cancer Res. 3, 1643-1651.

Niehans G.A., Singleton T.P., Dykoski D. and Kiang D.T. (1993). Stability of HER-2/neu expression over time and at multiple metastatic sites. J. Natl. Cancer Inst. 85, 1230-1235.

O'Grady A., Flahavan C.M., Kay E.W., Barrett H.L. and Leader M.B. (2003). HER-2 analysis in tissue microarrays of archival human breast cancer. Comparison of immunohistochemistry and fluorescence in situ hybridization. Appl. Immunohistochem. Mol. Morphol. 11, 177-182.

Olsen K.E., Knudsen H., Rasmussen B.B., Balslev E., Knoop A., Ejlertsen B., Nielsen K.V., Schönau A. and Overgaard J. (2004). Amplification of HER2 and TOP2A and deletion of TOP2A genes in breast cancer investigated by new FISH probes. Acta Oncol. 43, 35-42.

Paik S., Bryant J., Park C., Fisher B., Tan-Chiu E., Hyams D., Fisher E.R., Lippman M.E., Wickerham D.L. and Wolmark N. (1998). erbB-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J. Natl. Cancer Inst. 90, 1361-1370.

Pauletti G., Dandekar S., Rong H.M., Ramos L., Peng H.J., Seshadri R. and Slamon D.J. (2000). Assessment of methods for tissue-based detection of the HER-2 alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J. Clin. Oncol. 18, 3651-3664.

Penault-Llorca F. and Cayre A. (2004). Assessment of HER2 status in breast cancer. Bull. Cancer 91, S211-S215.

Perez E.A., Roche P.C., Jenkins R.B., Reynolds C.A., Halling K.C., Ingle J.N. and Wold L.E. (2002). HER2 testing in patients with breast cancer: poor correlation between weak positivity by immunohistochemistry and gene amplification by fluorescence in situ hybridization. Mayo Clin. Proc. 77, 148-154.

Popescu N.C., King C.R. and Kraus M.H. (1989). Localization of the human erbB-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32. Genomics 4, 362-366.

Quenel N., Wafflart J., Bonichon F., de Mascarel I., Trojani M., Durand M., Avril A. and Coindre J.M. (1995). The prognostic value of c-erbB2 in primary breast carcinomas: a study on 942 cases. Breast

Cancer Res. Treat. 35, 283-291.

Rhodes A., Jasany B., Anderson E., Dodson A.R. and Balaton A.J. (2002). Evaluation of HER-2 immunohistochemical assay sensitivity and scoring on formalin-fixed and paraffin-processed cell lines and breast tumors. A comparative study involving results from laboratories in 21 countries. Am. J. Clin. Pathol. 118, 408-417.

Ridolfi R.L., Jamehdor M.R. and Arber J.M. (2000). HER-2/neu testing in breast carcinoma: a combined immunohistochemical and fluorescence in situ hybridization approach. Mod. Pathol. 13, 866-873.

Ross J.S. and Fletcher J.A. (1998). The HER-2/neu oncogene in breast cancer: prognostic factor, predictive factor, and target for therapy. Oncologist 3, 237-252.

Schechter A.L., Stern D.F., Vaidyanathan L. Decker S.J., Drebin J.A., Greene M.I. and Weinberg R.A. (1984). The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen. Nature 312, 513-516.

Schlemmer B.O., Sorensen B.S., Overgaard J., Olsen K.E., Gjerdrum L.M. and Nexo E. (2004). Quantitative PCR – new diagnostic tool for quantifying specific mRNA and DNA molecules: HER/2 neu DNA quantification with LightCycler real-time PCR in comparison with immunohistochemistry and fluorescence in situ hybridization. Scand. J. Clin. Lab. Invest. 64, 511-521.

Schmidt M., Lewark B., Kohlschmidt N., Glawatz C., Steiner E., Tanner B., Pilch H., Weikel W., Kolbl H. and Lehr H.A. (2005). Long-term prognostic significance of HER-2/neu in untreated node-negative breast cancer depends on the method of testing. Breast Cancer Res. 7, 256-266.

Selvarajan S., Bay B.-H., Choo A., Chuah K.-L., Sivaswaren C.R., Tien S.-L., Wong C.-Y. and Tan P.-H. (2002). Effect of fixation period on HER2/neu gene amplification detected by fluorescence in situ hybridization in invasive breast carcinoma. J. Histochem. Cytochem. 50, 1693-1696.

Slamon D.J., Clark G.M., Wong S.G., Levin W.J., Ullrich A. and McGuire W.L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235, 177-182.

Slamon D.J., Godolphin W., Jones L.A., Holt J.A., Wong S.G., Keith D.E., Levin W.J., Stuart S.G., Udove J., Ullrich A. and Press M.F. (1989). Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244, 707-712.

Slamon D.J., Leyland-Jones B., Shack S., Fuchs H., Paton V., Bajamonde A., Fleming T., Eiermann W., Wolter J., Pegram M., Baselga J. and Norton L. (2001). Use of chemotherapy plus a monoclonal antibody against HER2. N. Engl. J. Med. 344, 783-792.

Stal O., Sullivan S., Wingren S., Skoog L., Rutqvist L.E., Carstensen J.M. and Nordenskjold B. (1995). c-erbB2 expression and benefit from adjuvant chemotherapy and radiotherapy of breast cancer. Eur. J. Cancer 31A, 2185-2190.

Tapia C., Schraml P., Simon R., Al-Kuraya K.S., Maurer R., Mirlacher M., Novotny H., Spichtin H., Mihatsch M.J. and Sauter G. (2004). HER2 analysis in breast cancer: reduced immunoreactivity in FISH non-informative cancer biopsies. Int. J. Oncol. 25, 1551-1557.

Thor A.D., Berry D.A., Budman D.R., Muss H.B., Kute T., Henderson I.C., Barcos M., Cirrincione C., Edgerton S., Allred C., Norton L. and Liu E.T. (1998). erbB-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. J. Natl. Cancer Inst. 90, 1346-1360.

Vogel C.L., Cobleigh M.A., Tripathy D., Gutheil J.C., Harris L.N., Fehrenbacher L., Slamon D.J., Murphy M., Novotny W.F., Burchmore M., Shak S., Stewart S.J. and Press M. (2002). Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J. Clin. Oncol. 20, 719-726.

Wixom C.R., Albers E.A. and Weidner N. (2004). HER2 amplification. Correlation of chromogenic in situ hybridization with immunohistochemistry and fluorescence in situ hybridization. Appl. Immunohistochem. Mol. Morphol. 12, 248-251.

Yaziji H. and Gown A.M. (2004). Accuracy and precision in HER2/neu testing in breast cancer: are we there yet? Hum. Pathol. 35, 143-146.

Zarbo R.J. and Hammond E.H. (2003). HER-2 testing of breast cancer patients in clinical practice. Arch. Pathol. Lab. Med. 127, 549-553.

Zeillinger R., Kury F., Czerwenka K., Kubista E., Sliutz G., Knogler W., Huber J., Zielinski C., Reiner G., Jakesz R., Staffen A., Reiner A., Wrba F. and Spona J. (1989). HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer. Oncogene 4, 109-114.