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HER2 status determination using RNA-ISH - a rapid and simple technique showing high correlation with FISH and IHC in 141 cases of breast cancer

Javier Alba[1], Javier Gutierrez[2], Victoria Mary Coupe[2], Beatriz Fernández[1], Ángel Vázquez-Boquete[1], Jesús Alba[2], Jerónimo Forteza[1] and Tomás García-Caballero[1]

[1]Department of Pathology, University Clinical Hospital, University of Santiago de Compostela, Santiago de Compostela, Spain and [2]CENBIMO, Lugo, Spain

Summary. Aims: the assessment of the human epidermic growth factor receptor 2 (HER2) is currently performed in most laboratories using two techniques: Fluorescence in situ hybridisation (FISH) and inmunohistochemistry (IHC), and novel methodology is being investigated continuously in the assessment of HER2, such as SISH, CISH, DNA chips, ELISA or real time PCR to make assessment easier, faster or more accurate. RNA-ISH (RNA in Situ Hybridisation) is a new technique designed to detect mRNA expression levels, conducted by light microscope without the need for counting or grading systems in a total processing time of 4 hours. This study aims to determine if RNA-ISH is a viable and effective technique and a possible alternative to the currently used techniques by analysing and comparing genetic amplification (FISH) and protein levels (IHC) with mRNA over-expression (RNA-ISH) in 141 cases of breast cancer. Results: This study demonstrated a 96.5% concordance between over-expression of HER2 as determined by RNA-ISH and gene amplification as determined by FISH. The relationship between RNA-ISH-evaluated and IHC-evaluated over-expression was equally well reflected with a 95.2% concordance. Importantly, a considerable reduction in processing and evaluation time was achieved of only 4 hours. Conclusions: We conclude that the probe developed for RNA-ISH represents a viable, effective possible alternative to FISH and IHC for analysing HER2 status in primary breast tumours.

Key words: RNA-ISH, FISH, ErbB2, HER2, Breast cancer, IHC

Offprint requests to: Dr. Javier Alba, Department of Pathology, University Clinical Hospital, University of Santiago de Compostela, Santiago de Compostela, Spain. e-mail: uscbiologist@gmail.com

Introduction

HER2 over-expression has been documented in approximately 15-23% (Walker et al., 2008) of breast carcinomas and is associated with worse tumour prognosis, resistance to conventional treatments and an increased probability of metastasis (Slamon et al., 1989; Baselga and Swain, 2009; Hynes and MacDonald, 2009; Kedrin et al., 2009). Over-expression of HER2 has also been associated with other types of tumours such as gastric carcinomas and small cell lung cancers (Hung and Lau, 1999; Baselga and Swain, 2009).

Trastuzumab (Herceptin®; Genetech, San Francisco, CA, USA), the principal treatment for HER2 positive breast tumors, is a humanized monoclonal antibody which is used as specific treatment against tumours that over-express HER2. Administration of the antibody produces considerable clinical improvement but can also provoke adverse side effects such as cardiac congestion and hypertension (Azim et al., 2009). Another treatment, Laptinib (Tykerb® Glaxo Smith Kline, Brentford, UK), is a tyrosine kinase inhibitor that interrupts HER2 receptor signalling (Menard et al., 2003; Ross et al., 2009; Roy and Perez, 2009).

A proper determination of HER2 status is essential to provide adequate treatment, and the American Society of Clinical Oncology / College of American Pathologists (ASCO/CAP) recommend the determination of HER2 status in all invasive breast cancers using IHC and then, contrast the equivocal results by ISH (Wolff et al., 2007).

Analysis by IHC is based on the recognition of the extracellular fraction of HER2 by a monoclonal or polyclonal antibody. The technique indicates levels of protein through a chromogenic reaction analysed by light microscope. Determination of over-expression is

achieved using a grading system 0, +1, +2, +3 reflecting the intensity and circumferential character of the staining. The routine and recommended practice in diagnostic laboratories is a primary screening by IHC in which 0 and +1 cases are considered as negative, +3 cases as positive and +2 cases as equivocal, requiring confirmation by ISH (Wolff et al., 2007).

To date, fluorescence in situ hybridisation is the ISH technique most frequently used in the determination of HER2 gene amplification and is regarded as the 'gold standard' (Sauter et al., 2009) its analysis is based on the recognition of a target sequence of denatured DNA by complementary fluorescence-labelled probe, and the procedure consists of counting and comparing the number of HER2 gene copies and number of chromosomes; a correlation greater than 2.2 implies gene amplification which is highly correlated with erbB2 overexpression.

Both IHC and FISH have advantages and disadvantages. IHC is fast in interpretation and technique performance. On the other hand, is not definitive, since the +2 must be analysed by another technique and there is some variation between laboratories.

FISH has the advantage of high reliability in the diagnosis, is very objective and does not need to be verified by another technique. Also in contrast it requires more time in the diagnosis and implementation of technology, also, contextualising fluorescence in the tissue is more complex and requires specialised equipment (Bhargava et al., 2005; Garcia-Caballero et al., 2010)

Owing to the importance of reliable diagnosis for HER2 expression due to high case volumes and the economic and social implications of treatment, there have been recent improvements and the development of new techniques to determine HER2 status such as real-time quantitative PCR, CISH or ELISA (Laudadio et al., 2007; Kurosumi, 2009; Ross et al., 2009).

RNA-ISH is a new hybridisation technique that uses probes composed of very long single stranded DNA fragments that are targeted to mRNA. Due to the length of these fragments it is necessary to raise the incubation temperature in order to achieve high specificity, and this increase has the additional beneficial effect of reducing the hybridisation time to just one hour. The RNA-ISH protocol can be completed in a total processing time of just 4 hours. RNA-ISH is a chromogenic technique; the labelled probe is recognised by enzyme-conjugated antibodies such as horseradish peroxidise (HRP), which can then be visualised by addition of diaminobenzidine (DAB). In this way mRNA can be detected by light microscope and the technique can be semi-automated resulting in very low effort by the technicians.

RNA-ISH analyses expression levels of HER2 mRNA, which is found in the cytoplasm. It does not use any type of grading system, as the appearance of cytoplasmic staining, independent of intensity, indicates a pathological increase in the expression of HER2.

In this study we have compared HER2 evaluation results from FISH with IHC and those resulting from the new in situ hybridisation against mRNA - RNA-ISH in 141 cases of primary breast cancer.

Materials and methods

Consecutive breast cancer tumours were selected. All techniques were performed and analysed in duplicate.

Tumour specimens

The study included 141 cases of breast cancer biopsies that were formalin fixed and paraffin embedded following routine protocols. 131 cases corresponded to ductal infiltrating carcinomas of different grades. The remaining 10 cases were varying tumours such as lobular invasive carcinomas. Tissue sections were cut to 4μm and mounted on in-house silanised microscope slides following routine procedures.

FISH

FISH was conducted using HER2 FISH pharmDX™ (Dako Denmark A/S, Glostrup, Denmark) probes. The assay was performed according to the manufacturer's recommendations and subsequently evaluated using a fluorescence microscope (Olympus BX51) equipped with appropriate filters for 4'-6 diamidino-2-phenylindole (DAPI), fluorescein isothiocynate (FITC) and Texas Red at 100X magnification.

RNA-ISH

RNA-ISH was conducted following the HistoSonda™ protocol (CENBIMO, Spain). The probe against HER2 developed by CENBIMO consists of two single stranded DNA fragments of 800 and 1000 base pairs which span approximately half of the mRNA sequence, assuring high specificity. The probe contains digoxigenin labelled uracils.

Tissue sections were de-waxed using the same protocol as FISH, after which peroxidase activity was blocked by submerging the slides in a solution of methanol /3% H_2O_2 for 5 minutes. Tissues were treated with Proteinase K (30 μg/mL) for 10 minutes at room temperature and subsequently washed for 3 minutes with distilled water and transferred to PBS pH 7.4. Excess PBS was removed from the tissue borders and 65 μl of probe solution was used to cover the section. Incubation of the probe was performed at 62°C for one hour in a closed humid chamber inside an incubator. After incubation the slides were vigorously washed with PBS pH 7.4, excess PBS removed and slides incubated for 30 min at room temperature with primary antibody anti-digoxigenin (Jackson, USA). Slides were then washed

with PBS and incubated 30 min at room temperature with secondary polymer antibody-HRP (Dako REAL™ EnVision kit, Dako) and washed with PBS. To visualise the digoxigenin-antibody conjugate the tissue section was covered with 50 μL of DAB at room temperature for 5 min. Counterstaining with haematoxylin was brief (2-3 sec, 50% diluted) and slides were then dehydrated and cover-slipped following normal procedures. The result was analysed by bright field microscope.

IHC

HercepTest (Dako) was used for IHC testing. The assay was performed according to the manufacturer's recommendations.

Scoring

Two different kinds of staining using RNA-ISH technique appear: nuclear and cytoplasmic staining. As indicated by protocol only the cytoplasmic staining was taken into account and nuclear staining was ignored. Any intensity of cytoplasmic staining was considered positive for HER2 over-expression. The evaluation was performed blind. Previous studies developed by this laboratory showed that nuclear staining is due to the presence of RNA retained in the nucleus and therefore incapable of producing protein (data not shown)

FISH was evaluated following instructions included in the Dako HER2 FISH pharmaDX™ kit. HER2 and Centromere 17 (CEN17) signals were counted in 20 nuclei and ratios HER2:CEN17 were calculated. If the ratio resulted as borderline (1.8-2.2), another 20 nuclei were scored from the same specimen recalculating the ratio for a total of 40 nuclei. Tumours that had a HER2:CEN17 ratio equal to or higher than 2.0 were considered as amplified; cases with a lower ratio were considered as normal.

The time required for the observer to evaluate each case of RNA-ISH, FISH and IHC was recorded.
IHC evaluation was conducted following ASCO / CAP recommendations (Wolff et al., 2007).

Ethics

No patient demographic data or medical history was collected and this investigation was performed in compliance with local regulations and the current version of the World Medical Association Declaration of Helsinki.

Results

RNA-ISH was conducted in 141 cases, as shown in Table 1. A total of 30 cases were positive for cytoplasmic staining of HER2 mRNA and 111 cases were negative. The positive cases showed different levels of intensity as illustrated in Figure 1.

Cytoplasmic staining was only observed in tumour cells. Normal mammary cells in the rest of the breast tissue were negative and no unspecific background staining was observed in any case.

Nuclear dot-like staining appeared in 46 cases but was ignored for the final study as described by the protocol, it appears in positive and negative cases for cytoplasmic staining as showed in Figure 2.

FISH vs RNA-ISH concordance

Amplified or normal HER2 status as assessed by FISH was assigned to the tissue sections based on the ratio HER2:CEN17.

As shown in Table 1, of the 30 cases that showed over-expression as demonstrated by RNA-ISH, 25 were gene amplified by FISH. This gives a correlation of 96.5% (K value=0.895) between the two techniques, and of 141 cases only 5 discordant cases were found (one of these cases is shown in Fig. 3).

IHC vs RNA-ISH concordance

Comparing between RNA-ISH and IHC as shown in Table 1, 28 cases were found to be 3+ and 59 negatives (0 and +1). The correlation between IHC and RNA-ISH (excluding +2 cases as it is known that 20% of these cases are in fact positive) was 95.2% (K value=0.893) (table 3).

The concordance between IHC (excluding +2 cases)

Table 1. HER2 status of 141 breast cancer cases as determined by RNA-ISH (positive when cytoplasmic staining is observed), FISH (positive if the HER2:CEN17 ratio was equal or higher than 2.0 and negative if it was lower than 2.0) and IHC (positive if scored +3, negative if scored 0 or +1 and uncertain if scored +2).

STATUS	RNA-ISH	FISH	IHC
POSITIVE	30	25	28
NEGATIVE	111	116	59
UNCERTAIN			54

Table 2. Crosstabulation of HER2 status by Fluorescence in situ Hybridisation (FISH) and Rapid in situ Hybridisation (RNA-ISH) in 141 cases of breast cancer.

	FISH		
	NORMAL	AMPLIFIED	Total
RNA-ISH			
NEGATIVE	111	0	111
POSITIVE	5	25	30
Total	116	25	141

Concordance 96.5 %; K value 0.895.

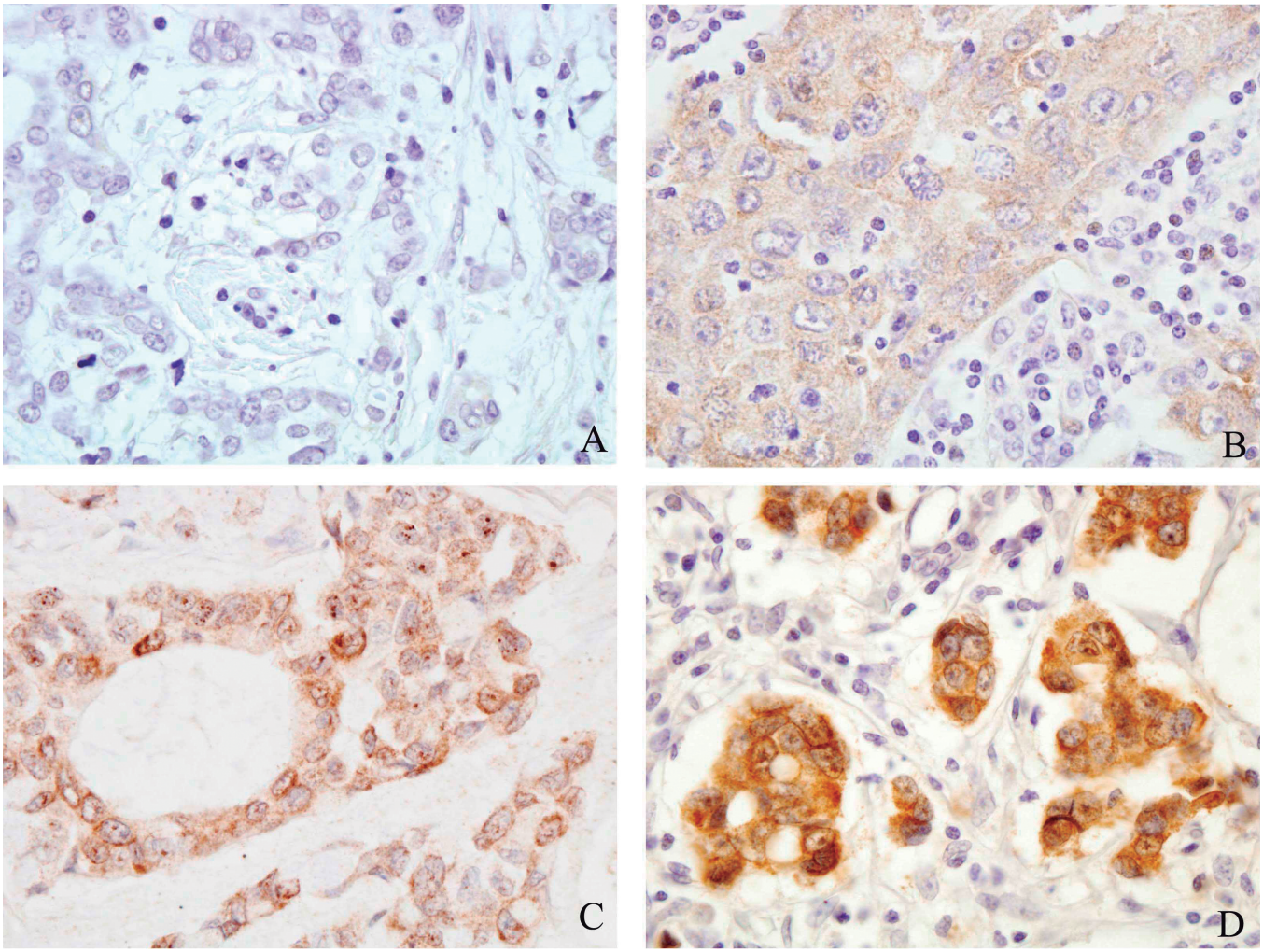

Fig. 1. Microphotographs from four HER2 breast cancer specimens stained with the RNA-ISH protocol showing different intensity in cytoplasmic staining in a negative case **(A)** and 3 positive cases: weak staining **(B)**, moderate staining **(C)** a and strong staining **(D)**. x 60

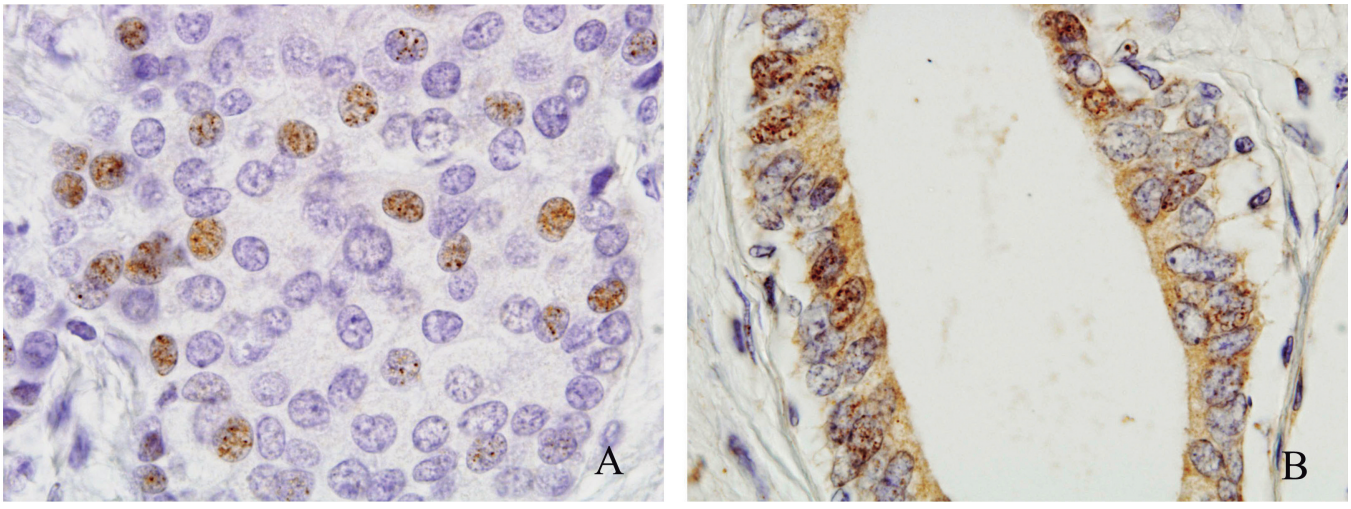

Fig. 2. Microphotographs from two HER2 breast cancer specimens stained with the RNA-ISH protocol showing nuclear dots and negative cytoplasmic staining **(A)** and nuclear dots and positive cytoplasmic staining **(B)**. The image on the left is clearly negative for cytoplasmic staining while the image on the right is positive. x 60

Table 3. Crosstabulation of HER2 status by immunohistochemistry (IHC) and Rapid in situ Hybridisation (RNA-ISH) in 87 cases of breast cancer.

	IHC		
	NEGATIVE	POSITIVE	Total
RNA-ISH			
NEGATIVE	57	2	59
POSITIVE	2	26	28
Total	59	28	87

Concordance 95.2%; K value 0.893.

and FISH was 97.1%(data not shown).

RNA-ISH vs. FISH vs. IHC scoring time

To compare scoring time for the RNA-ISH and the other two techniques, the time required for the observer to draw his evaluation from each method was measured. From this data a mean time of 4.9 min was calculated to evaluate genetic amplification by FISH vs. a mean time of 0.38 min for the evaluation of HER2 over-expression by RNA-ISH vs a mean time of 2.28 min to evaluate the intensity of the protein staining by IHC. This data shows that the evaluation time of HER2 status by FISH analysis is 2.14 times that necessary to evaluate protein expression and 12.9 times that necessary to evaluate

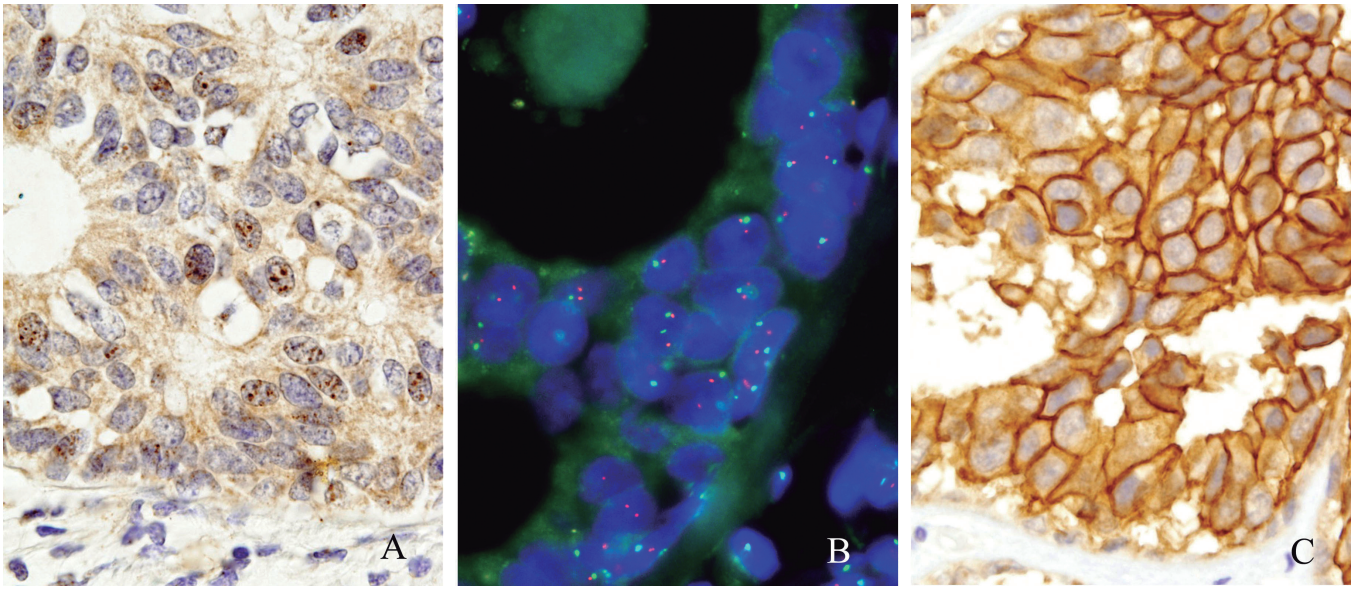

Fig. 3. Microphotographs of a discordant case between RNA-ISH and FISH. **A.** Positive RNA-ISH result. **B.** Negative FISH result. **C.** Positive (+3) IHC result. A, C, x 40; B, x 100

Table 4. Measurements of the time (in minutes) required for HER2 evaluation by RNA-ISH, FISH and IHC; mean scoring time was calculated.

N°	RNA-ISH	FISH	IHC
1	0.33	5.22	2.33
2	0.37	4.95	2.25
3	0.32	5.25	3.08
4	0.50	6.38	2.38
5	0.33	4.42	1.78
6	0.40	4.90	1.98
7	0.38	5.33	2.52
8	0.45	4.78	1.61
9	0.32	5.83	2.51
10	0.37	5.01	2.37
MEAN	0.377	5.207	2.281

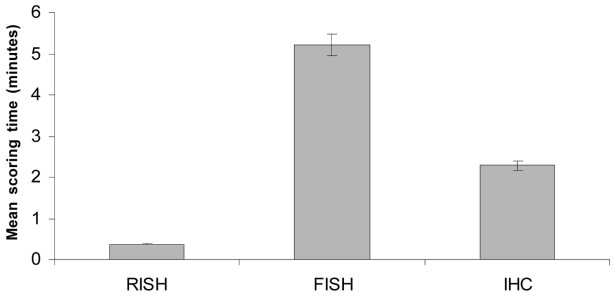

Fig. 4. The mean scoring times per slide for Rapid in situ hybridisation (RNA-ISH), Fluorescence in situ hybridisation (FISH) and immunohistochemistry (IHC) analysis. The 95% confidence interval is shown as the error bar.

cytoplasmic staining by RNA-ISH. Also the evaluation time of HER2 status by IHC is 6 times that necessary to evaluate RNA expression by RNA-ISH.

Discussion

Breast cancer is a worldwide health problem. In 2002 it was the cause of 410,000 deaths and was diagnosed in about 1,150,000 women in the entire world population. More developed societies have the greatest risk, with over 85 incidents per 100,000 women per year (data from the World Cancer report 2008).

To date, tumours that are shown to over-express HER2 are treated with trastuzumab or lapatinib, a tyrosine kinase inhibitor (Tykerb) (Menard et al., 2003; Ross et al., 2009; Roy and Perez, 2009). However, these drugs can provoke side effects (Azim et al., 2009) and represent a considerable increase in the cost of treatment - this combined with the high number of tumours analysed by anatomical pathology laboratories highlights the very real need for the use of a method that is both rapid, robust and reliable for HER2 status determination.

Detection target

HER2 status determination in breast carcinomas has previously been conducted by analysing numbers of gene copies using Southern Blotting, PCR and FISH / CISH and by analysing protein levels using immunoblotting, ELISA or IHC. However, a reliable method for the detection of mRNA levels has not existed until now and has only been possible by quantitative PCR (Bilous et al., 2003; Rosa et al., 2009; Savino et al., 2009).

It is obvious that for the detection of gene expression levels of a membrane receptor the principal target is the quantity of protein, but when antibody techniques show background or the determination is complicated or not definitive, like in the case of the HER2 protein, the assessment needs a new target, in this case the target which has been used so far is the DNA assessed by different techniques like CISH, FISH or PCR.

We think that the detection of RNA is a good alternative and in some aspects may even be advantageous to the detection of DNA copy numbers. It is known that there are ways of obtaining large quantities of gene expression without the need for a high number of gene copies (Kraus et al., 1987; Child et al., 1999). One such clear example is that of albumin produced by the liver (Hawkins and Dugaiczyk, 1982). In the same way a high number of gene copies does not always translate to high gene expression as shown by the example of ß-haemoglobin that has various copies of the gene but only one of those is functional (Wood, 1996).

Genetic expression does not definitively depend on number of gene copies, and although studies (this one included) have shown a strong correlation between over-expression and genetic amplification of HER2, tumour lines have been identified that over-express HER2 without gene amplification. It is possible that the discordant cases described in this study are due to tumours that over-expresses HER2 without genetic amplification, more studies are necessary to resolve this question.

On the other hand RNA is a weaker molecule compared with DNA, so RNA is more sensitive to procedures of fixation and deproteinization. When changes in fixation exist, like different hospital protocols or bad fixation due to tumour size, the deproteinization time needs to be changed. However, if a deproteinization protocol is set for these exceptional cases the technique will always give good results.

RNA-ISH Technology.

The greatest advantage of RNA-ISH lies in the speed of both the technique and its evaluation. The technique requires only 4 hours to be performed and the evaluation is much faster than that of FISH or IHC as the pathologist is able to determine HER2 over-expression in less than 1 minute without the need to grade or count. RNA-ISH is a semi-automatic procedure, after one hour of incubation with the probe (manual procedure), the technique consists of a typical inmunohystochemistry protocol with a primary antibody against digoxigenin (automatic procedure).

RNA-ISH is a bright microscope technique, so it is easy for the pathologist to contextualise the tumoural cells in the tissue, and this may suppose an advantage over PCR techniques which are not normally used in histopathology laboratories for HER2 status determination.

HER2 determination algorithm

The ASCO/CAP have determined that the first technique that should be done is immunohistochemistry and equivocal cases should be resolved by an ISH technique. Although this process demonstrates good results, some new articles have given rise to some controversy, suggesting that FISH should be performed first. With this article we show a new technique capable of studying a previously infrequently used target, mRNA, which shows a high correlation with the other two more used techniques.

While we do not want to propose this technique as a substitute for either of the two techniques (more studies are needed on the response to treatment after diagnosis with RNA-ISH which are being conducted in several centers in Europe), we think that due to the high correlations demonstrated by RNA-ISH, the new detection target and the rapid protocol, it could be proposed as a new alternative in HER2 status diagnosis.

References

Azim H., Azim H.A. Jr and Escudier B. (2009). Trastuzumab versus lapatinib: the cardiac side of the story. Cancer Treat. Rev. 35, 633-

638.

Baselga J. and Swain S.M. (2009). Novel anticancer targets: revisiting ERBB2 and discovering ERBB3. Nat. Rev. Cancer 9, 463-475.

Bhargava R., Lal P. and Chen B. (2005). Chromogenic in situ hybridization for the detection of HER-2/neu gene amplification in breast cancer with an emphasis on tumors with borderline and low-level amplification: does it measure up to fluorescence in situ hybridization? Am. J. Clin. Pathol. 123, 237-243

Bilous M., Dowsett M., Hanna W., Isola J., Lebeau A., Moreno A., Penault-Llorca F., Rüschoff J., Tomasic G. and van de VIjver M. (2003). Current perspectives on HER2 testing: a review of national testing guidelines. Mod. Pathol. 16, 173-182.

Child S.J., Miller M.K. and Geballe A.P. (1999). Cell type-dependent and -independent control of HER-2/neu translation. Int. J. Biochem. Cell. Biol. 31, 201-213.

Garcia-Caballero T., Grabau D., Green A.R., Gregory J., Schad A., Kohlwes E., Ellis I.O., Watts S. and Mollerup J. (2010). Determination of HER2 amplification in primary breast cancer using dual-colour chromogenic in situ hybridization is comparable to fluorescence in situ hybridization: a European multicentre study involving 168 specimens. Histopathology 56, 472-480.

Hawkins J.W. and Dugaiczyk A. (1982). The human serum albumin gene: structure of a unique locus. Gene 19, 55-58.

Hung M.C. and Lau Y.K. (1999). Basic science of HER-2/neu: a review. Semin. Oncol. 26, 51-59.

Hynes N.E. and MacDonald G. (2009). ErbB receptors and signaling pathways in cancer. Curr. Opin. Cell. Biol. 21, 177-184.

Kedrin D., Wyckoff J., Boimel P.J., Coniglio S.J., Hynes N.E., Arteaga C.L. and Segall J.E. (2009). ERBB1 and ERBB2 have distinct functions in tumor cell invasion and intravasation. Clin. Cancer. Res. 15, 3733-3739.

Kraus M.H., Popescu N.C., Amsbaugh S.C. and King C.R. (1987). Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J. 6, 605-610.

Kurosumi M. (2009). Recent trends of HER-2 testing and trastuzumab therapy for breast cancer. Breast Cancer 16, 284-287.

Laudadio J., Quigley D.I., Tubbs R. and Wolff D.J. (2007). HER2 testing: a review of detection methodologies and their clinical performance. Expert. Rev. Mol. Diagn. 7, 53-64.

Menard S., Pupa S.M., Campiglio M. and Tagliabue E. (2003). Biologic and therapeutic role of HER2 in cancer. Oncogene 22, 6570-6578.

Rosa F.E., Silveira S.M., Silveira C.G., Bergamo N.A., Neto F.A., Domingues M.A., Soares F.A. Caldeira J.R. and Rogatto S.R. (2009). Quantitative real-time RT-PCR and chromogenic in situ hybridization: precise methods to detect HER-2 status in breast carcinoma. BMC Cancer 9, 90.

Ross J.S., Slodkowska E.A., Symmans W.F., Pusztai L., Ravdin P.M. and Hortobagyi G.N. (2009). The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. Oncologist 14, 320-368.

Roy V. and Perez E.A. (2009). Beyond trastuzumab: small molecule tyrosine kinase inhibitors in HER-2-positive breast cancer. Oncologist 14, 1061-1069.

Sauter G., Lee J., Bartlett J.M., Slamon D.J. and Press M.F. (2009). Guidelines for human epidermal growth factor receptor 2 testing: biologic and methodologic considerations. J. Clin. Oncol. 27, 1323-1333.

Savino M., Parrella P., Copetti M., Barbano R., Murgo R., Fazio V.M., Valori V.M., Carella M., Garrubba M. and Santini S.A. (2009). Comparison between real-time quantitative PCR detection of HER2 mRNA copy number in peripheral blood and ELISA of serum HER2 protein for determining HER2 status in breast cancer patients. Cell. Oncol. 31, 203-211.

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.

Walker R.A., Bartlett J.M., Dowsett M., Ellis I.O., Hanby A.M., Jasani B., MIller K. and Pinder S.E. (2008). HER2 testing in the UK: further update to recommendations. J. Clin. Pathol. 61, 818-82.

Wolff A.C., Hammond M.E., Schwartz J.N., Hagerty K.L., Alfred D.C., Cote R.J., Dowsett M., Fitzgibbons P.L., Hanna W.M., Langer A, McShane L.M., Plk S., Pegram M.D., Perez E.A., Press M.F., Rhodes A., Sturgeon C., Taube S.E., Tubbs R., Vance G.H., van de Vijver M., Wheeler T.M. and Hayes D.F. (2007). American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. J. Clin. Oncol. 25, 118-145.

Wood W.G. (1996). The complexities of beta globin gene regulation. Trends. Genet. 12, 204-206.

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