Evaluation of HER-2/*neu* Gene Amplification by Fluorescence *In Situ* Hybridization and Immunohistochemistry in Saudi Female Breast Cancer

HEBA AL-KHATTABI[1], ABDELHAKEEM KELANY[2], ABDELBASET BUHMEIDA[1], JAUDAH AL-MAGHRABI[3], SAHIRA LARI[4], ADEEL CHAUDHARY[1], MAMDOOH GARI[1], ADEL ABUZENADAH[1] and MOHMMAD AL-QAHTANI[1]

[1]Centre of Excellence in Genomic Medicine Research, KFMRC, [2]Department of Medical Biology, [3]Department of Pathology and [4]Department of Biochemistry, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia

Abstract. *Background: Amplification of the HER-2/neu oncogene and concomitant over-expression of its protein are detected in approximately 18% of invasive ductal carcinoma of the breast and is associated with poor prognosis. This study tested the use of fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) to evaluate the HER-2/neu gene status and to ascertain the concordance rate between the two methods. Patients and Methods: Eighty two tumour samples containing representative tumour were divided for testing using each assay. HER-2/neu gene amplification is scored as a ratio of HER-2/neu gene amplification to chromosome 17. The ratio should be >2.2 to be considered as positive. 20 cells should be counted and an average score taken. An extra 20 cells should be counted if the ratio is between 1.8-2.2. Results: Seventy five effective samples were used. HER-2/neu gene was amplified in 19 out of 75 cases (25%) whereas, HER-2 protein, by IHC was over-expressed in 18 out of 75 cases (24%). In the 44 negative cases by IHC analysis only 7 cases (16%) of them showed amplification by FISH. Three out of 13 cases (23 %) scored as +2 showed gene amplification by FISH while 9 cases out of 18 cases (50%) were scored as +3. High concordance with FISH results 37:44 (84 %) was noted in negative cases (0/+1 cases), while lower concordance 3:13 (23 %) was seen in +2 cases. Conclusion: This study revealed a significant concordance between FISH results and IHC results. The study also showed that HER2/neu amplification is higher in Saudi patients than other western populations. However, due to the inherent failures of the IHC assay, FISH should always be used when the IHC results are inconclusive. The rational algorithm for HER-2/neu testing would be to perform IHC first, followed by FISH to validate equivocal IHC results.*

Correspondence to: Dr. Abdelbaset Buhmeida, MD, Ph.D., Center of Excellence in Genomic Medicine Research (CEGMR). King Abdul-Aziz University. P.O. Box: 80216 Jeddah 21589, Saudi Arabia. Tel: +9662 6401000 Ext: 25481, Fax: +9662 6952521, e-mail: abuhmeida@kau.edu.sa, abuhme@utu.fi

Key Words: Concordance, FISH, IHC, HER-2/neu, breast cancer.

The heterogeneous disease group of breast cancer and its molecular subtypes has been characterised by many recurrent genetic abnormalities such as unbalanced chromosomal rearrangements, deletions and gene amplifications (1-3). A cursory literature review readily shows that the treatment of these cancers is assuming a special status befitting to the biological characteristics of the tumour rather than its clinico-pathological features (4-6). Therefore, gene amplification and protein expression are in sharp focus, as never before, for a genuine diagnosis of breast cancer, and in particular the search for HER-2 assay directed at breast cancer pathology (7).

The accuracy in HER-2/*neu* results is critical for the use of targeted therapies (8-10). In validation experiments, a close association between the HER-2/*neu* gene amplification and protein expression has been documented earlier by Western and Northern blot analysis (11). However, in contrast, in immunohistochemistry (IHC), deparaffinised - formalin - fixed tissue can be quite variable in its ability to identify HER-2/*neu* amplified tumours. A serious disparity also exists between HER-2 protein expression evaluated by IHC and fluorescence *in situ* hybridization (FISH) (12, 13). Conflicting trends have continued, even in recent studies, despite efforts to narrow the discrepancy between the two methods (14). For tumours that are moderately positive by IHC, further evaluation by FISH, staining 2+ by IHC instituting therapy is recommended (15).

One of the reasons for the discrepancy between the use of IHC analysis and FISH may arise from the use of formalin fixation, causing destruction of the HER-2 epitope thus

0250-7005/2010 $2.00+.40

leading to a false - negative IHC result in approximately 15%-20% of HER-2/*neu*-positive cases. FISH may assist in overcoming certain misclassification errors that can occur as a result of the tissue fixation procedure used in IHC. A significant decrease in false-positive (IHC 3^+/FISH$^-$) results has been indicated through the introduction of an FDA approved scoring system for HER-2/*neu* IHC using a normalised IHC score in breast cancer (16). Even when used alone, FISH is advantageous in that it is able to circumvent antigenic changes that occur in formalin fixed - paraffin embedded tissue; a major limitation of the IHC protocol. Unlike IHC, the use of FISH is limited by its relative expense, although it may be the overall more cost-effective option, since it is sensitive and highly specific and has the added advantage of being associated with most objective scoring system (17).

Accuracy can further be enhanced by using modified FISH, incorporating a more objective detection of HER-2/*neu* gene amplification in tissue by a greater technical precision (18). However, a key improvement, to both IHC and FISH, is the use of microscopic analysis, allowing assessment of changes by gene copy number and gene product expression (19).

It is evident that there is an urgent need to re-evaluate the IHC scoring method, IHC being still the widely used assay in most diagnostic centres. Also, immediate efforts must be made to narrow the gap between IHC and FISH assessment. Such studies are rare in the Saudi Arabia, despite initial reports indicating the serious rise in breast cancer cases (20). The aim of this study was to evaluate the HER-2/*neu* gene status in Saudi patients and furthermore to ascertain the concordance rate between FISH and IHC techniques.

Patients and Methods

Patient population and tissue specimens. Breast cancer tissue samples from 82 randomly selected patients, aged between 24-81 years reporting for breast cancer complications at King Abdul Aziz University Hospital, Jeddah, during 2002-2008 were used for the study, although due to technical reasons, only samples from 75 patients were finally included. All samples, preserved as paraffin blocks, were subjected to screening for HER-2/*neu* over-expression by immunohistochemical testing. Normal tissues were obtained from normal breast material for use as two negative controls. In each case, a section of 3-4 μm was excised from the same paraffin block of tumour to be used for IHC as well as in FISH, later. Only those sections having representative invasive cancer were used in the FISH study. Exposure of the specimen to acids, bases, or heat, was carefully avoided to minimise any damage to DNA leading to failure in FISH assay.

HER-2/ neu FISH on paraffin section

Tissue de-paraffinisation. Slides were immersed in xylene for 10 min at room temperature; this step was repeated twice using fresh xylene each time, followed by dehydrating in 100% ethanol with two immersions of 5 min each; the slides were later air dried. These were subsequently immersed in 0.2 M HCl for 20 min and transferred to purified water and washed in buffer (2 × SSC, pH 7) for 3 min. Thereafter the samples were immersed in pretreatment solution at 80˚C for 30 min. A volume of 50 ml of this solution was prewarmed at 80±1˚C in a water bath, after which the slides were immersed in purified water for 1 min followed by immersion in wash buffer for 5 min, twice, to complete the washing. Excess buffer was removed by blotting edges of the slide on a paper towel and then the slides were immersed in protease solution at 37˚C for 15 to 20 min; the pH of the protease buffer was kept between 0.8 - 1.5. The protease solution was prepared by adding 250 mg protease freshly before slide immersion to protease buffer, at 37±1˚C. Slides were immersed twice in wash buffer for 5 min and allowed to air dry.

The slides were evaluated for the ability to assess appropriate protein digestion for some cases before moving on to hybridisation. Variable time intervals were enforced as some tissues were resistant to digestion. After incubating the slides in protease for 15 to 20 min, the slides were rinsed in 2 × SSC buffer for 10-20 s, then 10 μl of DAPI counter stain was added to the slides and was replaced the coverslip. The slides were viewed using DAPI filter (Illinois, USA) and the tissue section assessed for under-, appropriate- and over-digestion. Cells that were over-digested appeared ghostly and showed loss of cell border, and for them, digestion was repeated with new slides at a reduced time; those that were under-digested were gently wiped off the immersion oil and the cover slip was removed by soaking the slide in 2 × SSC buffer. The slides were transferred to fresh 2 × SSC buffer for some time to clean off any residual oil. Should further digestion be required, protease treatment was repeated, readjusting the time of treatment.

The prepared slides were treated with neutral buffered formalin (4% formaldehyde in PBS) at room temperature for 10 min followed by two washing steps in wash buffer of 5 min each and air dried before processing for hybridisation. All the steps were carried out under continuous shaking and at room temperature unless otherwise stated.

FISH protocol. The pH of the denaturation solution, formamide being critical, was maintained at pH 7.0-7.5 at room temperature and for use, transferred to an appropriate container kept in a water bath at 73±1˚C. DNA was denatured by immersing the prepared slides for 5 min in this solution, after checking that the temperature was at 73±1˚C, and then transferred to 70% ethanol for 1 min, agitated to remove excess formamide, dehydrated in graded ethanol, 70% to 85% and 100%, for a 1 min dipping in each change. Excess ethanol was drained off and the slides wiped and air dried ready to be used for probe labelling.

A PathVysion (Abott Park, Illinois, USA) HER-2 DNA probe kit was used. The probe is composite, being composed of two probes; one LSI HER-2/*neu* DNA probe, with a 190 kb Spectrum Orange labelled with fluorescent DNA probe specific for the HER-2/*neu* gene locus (17q11.2-q12) and the other, a CEP 17 DNA probe; a 5.4 kb Spectrum Green labelled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1). Both probes were pre-mixed and pre-denatured in hybridisation buffer, needing just a mix and spin before use. The areas to be hybridised were marked with a diamond - tipped scribe. A volume of 10 μl of probe mixture was added to the target area of the slide. A 22 mm × 22 mm glass coverslip was placed immediately over the probe and the probe mixture was allowed to spread evenly under the coverslip. Air bubbles were avoided since they interfere with hybridisation. The cover slips were sealed with rubber cement and placed in the pre-warmed HYBrite

Table I. *Incidence of HER-2 over-expression by IHC and amplification by FISH.*

		No. of patients (%)
IHC score	0/+1	44 (59%)
	+2	13 (17%)
	+3	18 (24%)
	Total	75
FISH score	Non amplified	56 (75%)
	Amplified	19 (25%)
	Total	75

Table II. *Comparison f Her-2 status of breast carcinoma by FISH and IHC assays.*

FISH Score	IHC Score			
	(0/+1)	+2	+3	Total
Non amplified	37	10	9	56
Amplified	7	3	9	19
Total	44	13	18	75
Concordance (%)	84.1	23.1	50.0	

(Illinois, USA) at 37˚C for 16 h overnight, to allow hybridization to take place.

After removing the seals the slides were immersed in post hybridization wash buffer (2 × SSC/ 0.3% NP-40) at room temperature; the cover slip floated off, excess fluid was removed, and slides were immersed for 2 min in post hybridisation wash buffer kept at 73±1˚C. Each slide was air dried in the dark and then 10 μl of 4,6 diamidino 2- phenylindole (DAPI) counterstain was applied to the target area of the slide and re-covered by a cover slip and stored in the dark at 4˚C prior to signal enumeration and at −20˚C for long-term storage.

Fluorescence microscopy and interpretation of FISH results. The slides were subjected to fluorescence microscopy after gradually reaching room temperature. A total of 20 nuclei were enumerated and analysed according to the method described by Press *et al*. (21). The signals were carefully examined using Axioskop (Jena, Germany) and Axioplan fluorescence microscopes (Jena, Germany) equipped with 100 watt mercury lamps, and narrow band pass filters (Vysis, USA). Images were captured by analogue camera. The depth of focus was adjusted and scanned at 25 × objective to view the hybridised area and the relocated target of interest was viewed at 100 × objective. Analysis commenced in the upper left quadrant of the selected area and scanned from left to right. The numbers of signals were counted within the nuclear boundary of each evaluable inter-phase cell. Areas of necrosis and nuclear border ambiguities were excluded from the count. Similarly, nuclei with signals requiring subjective judgment or having weak intensity and non-specificity and with noisy background or having insufficient counter stain were excluded. Nuclei with no signals or with signals of only one colour were also excluded; only those nuclei showing discrete signals were enumerated. Focus was given to those nuclei with one or more FISH signals of each colour. Split signals were counted as a single hybridization signal. The numbers of LSI Her2/*neu* and CEP 17 signals per nucleus were recorded in columns. Results on counting of 20 inter-phase nuclei from tumour cells per target were reported as the ratio of the total Her2/*neu* signals to those of CEP 17. Specimens with amplification showed a LSI Her2/*neu*: CEP 17 signals ratio ≥2.2, whereas normal specimens showed a signal ratio of ≤1.8. Results at or near the cut-off point (1.8-2.2) should be interpreted with caution. In the event of a borderline result (1.8-2.2), particularly if there also appears to be variability of the counts from one nucleus to the other, 20 additional nuclei would be enumerated. The specimen slide was also re-enumerated by another technician to verify the results.

The ploidy status of chromosome 17 was determined by calculating the average number of copies of CEP 17 based on 20 cell nuclei. If the ratio of chromosome 17 to cell number was >2.25 this indicated that there were extra copies of chromosome 17, and if the ratio was ≤2 this indicated that there was an absence of chromosome 17 (22).

HER-2 immunohistochemistry. Immunohistochemistry was performed using the HercepTest as per the manufacturer's guidelines. The percentage and intensity of cells showing complete membrane staining was recorded. Cytoplasm staining was not included when interpreting the results. Her-2/*neu* protein expression was scored according to the following criteria: 0 indicated that no membrane staining was observed; +1 indicated faint partial membrane staining >10% of cancer cells; +2 indicated weak circumferential membrane staining in >10% of cancer cells but with a thin membrane staining ring and +3 indicated intense circumferential membrane staining in >10% of cancer cells and a thick membrane staining ring (chicken wire appearance).

Results

Of the 82 breast cancer patients, only 75 patients were included in the study. Seven cases were excluded due to technical reasons including: inappropriate hybridisation signals such as tissue fell off the slides; and in a few cases, fewer than 20 nuclei showed both hybridisation signals. The mean age of the patients was 52 years and the standard deviation was 13.14. In 12 out of 19 cases amplified by FISH the age of the patient was under the mean age of 52, and the remaining 7 patients were over 52 years of age.

Incidence of HER-2/*neu* gene amplification and its protein over expression is shown in Tables I and II. The HER-2/*neu* gene was amplified in 19 out of 75 cases (25%) whereas, the HER-2 proteins, by IHC was over-expressed in 18 out of 75 cases (24%). In the 44 negative cases by IHC, only seven cases (16%) showed amplification by FISH. Three out of 13 cases that were scored as +2 showed gene amplification by FISH and 9 of 18 cases that were amplified by FISH scored as +3. High concordance with FISH results, 37:44 (84%), was noted in negative cases (0/+1 cases) (84%), while lower concordance 3:13 (23%) was seen in +2 cases. These results support the argument that FISH provides a more accurate and

precise measure of HER-2 expression than IHC assays in formalin-fixed biopsies. Table III shows a comparative study of IHC and FISH in breast carcinomas for hypodysomy and polysomic 17, representing <2 and ≥2.25 copies per cell.

Non amplified and amplified signals of HER-2/*neu* gene with polysomy 17 during various observations are shown in Figure 1. Figure 2 shows the high signals monosomy 17 in non-amplified HER-2/*neu* and polysomy 17 without gene amplification.

The study further revealed that polysomy, hypodisomy and gene amplification can be detected accurately in individual cells by using FISH. Thus, a net increase in HER-2/*neu* gene copy number consecutive to polysomy 17 in the absence of specific gene amplification lead to a strong protein overexpression in a small subset of breast carcinoma observed in the present study.

Table III. *Incidence of hypodisomy 17 (chromosome 17<2 copies per cell) and polysomy 17(chromosome 17>3 copies per cell) in comparison with IHC and FISH in breast carcinoma.*

Chromosome 17	FISH Score	IHC Score		
		+1	+2	+3
Hypodisomy	Non amplified	27	4	7
	Amplified	-	1	-
	Total cases of aneusomy 17	27	5	7
Polysomy	Non amplified	2	5	2
	Amplified	4	2	3
	Total cases with polysomy 17	6	7	5

Discussion

Incidence of breast cancer among Saudi women is very high, prompting this study which investigated the role of genetic abnormality in such cases of Saudi breast cancer patients. Prior studies (23) have reported that HER-2/*neu* gene amplification is more common in younger patients, and the present study highlighted a significant relationship between HER-2/*neu* gene amplification and young patient age.

Considerable molecular differences from various ethnic groups are frequently reported. In one such study performed to identify the potential differences between breast cancer with HER-2/*neu* amplification between Swiss and Saudi women, a higher incidence of grade 3 cancer with complete absence of low grade cancer and higher incidence of HER-2/*neu* amplification has great implications for Saudi women (24). The variation is possibly related to a difference in genetic susceptibility and lifestyle of the two ethnic groups. In Saudi study, the results have showed that Saudi women also have high incidence of higher grade cancer and HER-2/*neu* amplification.

Indications are that HER-2/*neu* amplification significantly infiltrates ductal carcinoma more than lobular carcinomas and that higher grade tumours are more likely to demonstrate HER-2/*neu* amplification than lower grade carcinomas (25). That study also demonstrated that all cases with HER-2/*neu* amplification were infiltrating ductal carcinomas and they were of high grade.

Importantly, the current study showed that the prevalence of HER-2/*neu* amplification is slightly higher in Saudi patients as compared to other published studies (26). This prevalence rate was almost similar to a Qatari study (23), which revealed that the provenance rate of HER-2/*neu* gene is over-expressed in 26% of Qatari breast cancer patients. However, the small sample size of Qatari study might not accurately reflect the true prevalence.

The use of the FISH technique not only helps to assess the HER-2/*neu* gene status but further permits the determination of the number of copies of the gene and their spatial distribution within the nuclei (27) and also to define the exact level of HER-2/*neu* gene amplification by dual colour FISH analysis (28). As such, FISH has gained considerable interest as a reliable and valid method for determining HER-2/*neu* status, and its prognostic utility has been discussed by many authors (29). Unlike other gene-based assays, Southern blotting or polymerase chain reaction, FISH is not hampered by dilution artefacts. Notwithstanding the substantial cost of FISH, it is being considered as gold standard for assessing HER-2/*neu* status (30).

There is however the potential to obtain more reliable results by adjusting incubation times and checking protease treatment at the appropriate stage, as demonstrated for the first time in the present study. Although cases with high levels of gene amplification consistently show a gain of chromosome 17, between three and five copies per nucleus (31), this is in contrast to the low and intermediate levels of HER-2/*neu* gene amplification, showing lower gains of chromosome 17 and DNA aneuploidy. However, a small proportion of the cases also reveal unamplified HER-2/*neu* gene with monosomy 17, as described elsewhere (32).

The low frequency of the inverse association of monosomy 17 and HER-2/*neu* gene amplification are both of great interest (32). Concordance rates as high as 80-90% have been reported in the detection of protein overexpression in immunohistochemistry with gene amplification (FISH) (16). In spite of the good correlation between HER-2/*neu* gene amplification and protein overexpression, 3% to 15% of breast carcinomas overexpress the HER-2/*neu* protein without gene amplification and a small subset of breast carcinoma amplify the HER-2/*neu* gene without overexpression (29, 33). In the present study, HER-2/*neu* gene amplification was consistently found to be associated with protein overexpression, except in cases scored as

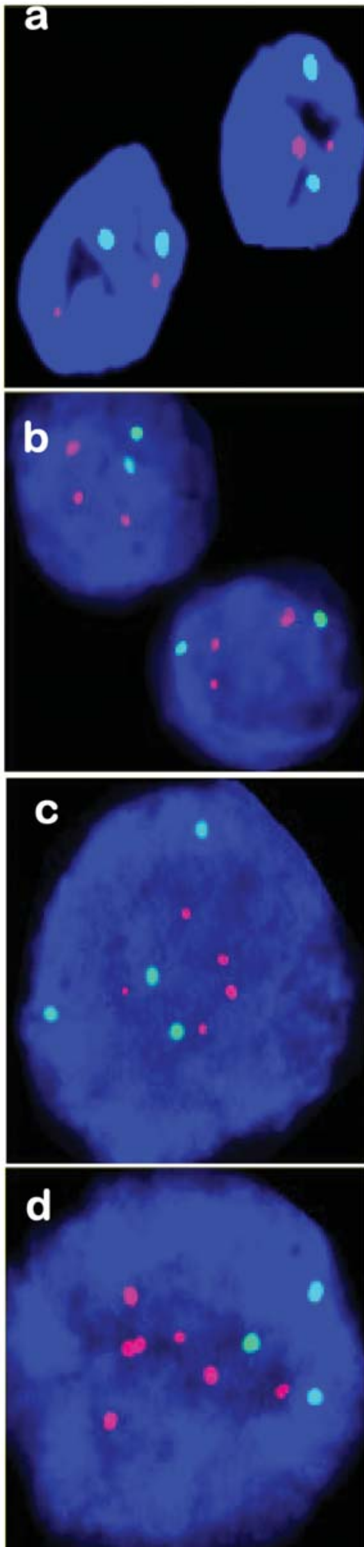

Figure 1. *Various levels of amplifications of HER-2 gene: (a) non amplified, where two green signals represented normal level of chromosome 17 and red signals represented normal level of HER-2 gene, (b) low level amplification without polysomy 17 and, (c) and (d); intermediate amplification in HER-2 gene with polysomy 17.*

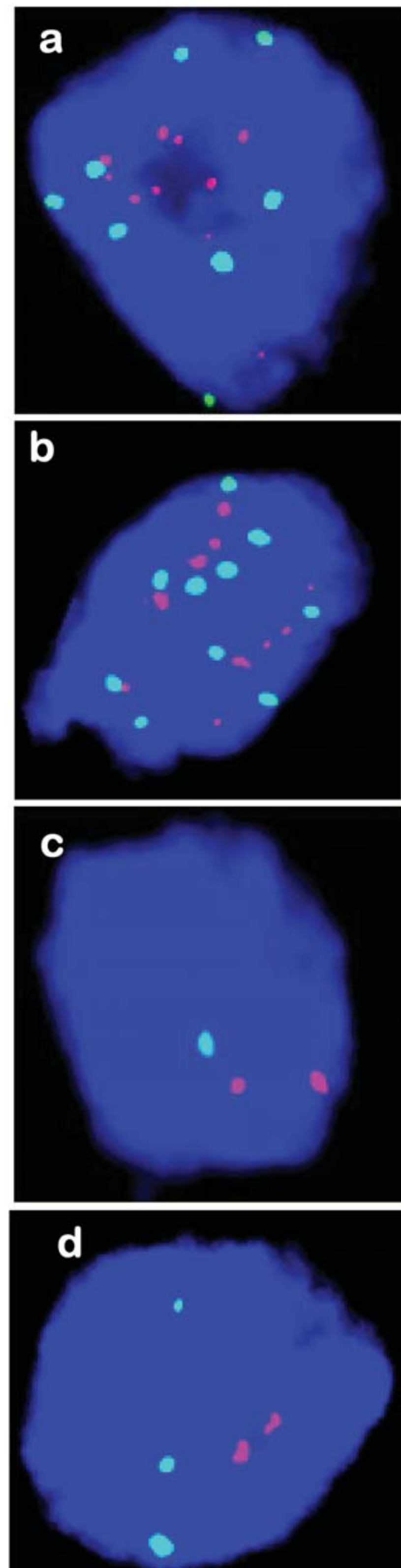

Figure 2. *Strongly amplified signals (a, b); and the corresponding monosomy 17 in non-amplified HER- 2 (c) and polysomy 17 without gene amplification (d).*

weakly positive. A few studies have failed to detect oncogene amplification by FISH of tumours with strong 3+ staining (36). Because overexpression of HER-2 is not necessarily caused only by amplification, polysomy of chromosome 17 has to be taken into account (37). This polysomy of chromosome 17 may well lead to an incorrect diagnosis of a low-level amplification detect

The earlier use of humanised anti-HER-2/*neu* monoclonal antibodies for precise identification (34) has found favour by other authors (35). Since the rationale for using weak expression for eligibility in trastuzumab treatment has been questioned, the therapy is primarily recommended for tumours that scored as 3+ by immunohistochemistry (15).

A study was submitted to the FDA in 2003 to validate FISH as a routine screening test for selection of patients for trastuzumab treatment, regardless of immunohistochemical score, and for patients with immunohistochemical scores of 3+ regardless of FISH scores (36). The FISH screening, as observed by (37) was found to be a better predictor of prognosis and trastuzumab eligibility than IHC. It excludes the majority of cases with immunohistochemical scores of 2+, having a minimal response to trastuzumab treatment (38). The present findings are based on two FDA approved tests for HER-2 detection and although they corroborate previous findings, some results did show fluctuation. This is related to the validity of the scoring systems of the current assays. The current study supports the concept that some of 2+ HercepTest cases result from artifactual sensitive staining and do not necessarily indicate gene amplification or polysomy 17. The current authors tend to agree with Slamon *et al*. (36). The study also showed that HER2/*neu* amplification is high in Saudi patients and therefore these results should be considered when subjecting patients whose tumours show weak overexpression of HER-2/*neu* by the HercepTest to the potential cardiac toxic effects of trastuzumab. Besides additional clinical studies that correlate trastuzumab response and prognosis of +2, FISH positive cases and +3, FISH negative cases, especially in relationship to polysomy17, should be investigated.

Conclusion

Considering the overall logistical difficulties, as well as the accuracy, time and cost for the double testing of HER-2/*neu* (IHC/FISH), gene analysis may be an efficient and useful approach for HER-2/*neu* screening of breast cancer patients, particularly for laboratories running a large number of breast cancer surgical specimens, where the pathological experience of the staff would guarantee a correct tumour grading. Importantly, this study showed that HER-2/*neu* amplification is slightly higher in Saudi patients as compared to other ethnic groups (26). There was a significant concordance between FISH results and IHC results. However, due to the inherent failures of the IHC assay, FISH should always be used when the IHC results are inconclusive. The rational algorithm for HER-2/*neu* testing would be to perform IHC first, followed by FISH to validate equivocal IHC results.

Acknowledgements

The authors would like to thank the laboratory help rendered by the technical staff at the Center of Excellence in Genomic Medicine Research Center (CEGMR), King Abdul-Aziz University, Jeddah. Many thanks are also extended to KACST, Riyadh and KAU, Jeddah, Saudi Arabia for supporting and generously funding this project.

Disclosure/Conflict of Interest

The authors have no conflicts of interest to declare. No part of this report has been presented elsewhere.

References

1 Chin K, DeVries S, Fridlyand J, Spellman PT, Roydasgupta R, Kuo WL, Lapuk A, Neve RM, Qian Z, Ryder T, Chen F, Feiler H, Tokuyasu T, Kingsley C, Dairkee S, Meng Z, Chew K, Pinkel D, Jain A, Ljung BM, Esserman L, Albertson DG, Waldman FM and Gray JW: Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. Cancer Cell *10*: 529-541, 2006.

2 Hwang ES, DeVries S, Chew KL, Moore DH, Kerlikowske K, Thor A, Ljung BM and Waldman FM: Patterns of chromosomal alterations in breast ductal carcinoma *in situ*. Clin Cancer Res *10*: 5160-5167, 2004.

3 Jones C, Ford E, Gillett C, Ryder K, Merrett S, Reis-Filho JS, Fulford LG, Hanby A and Lakhani SR: Molecular cytogenetic identification of subgroups of grade III invasive ductal breast carcinomas with different clinical outcomes. Clin Cancer Res *10*: 5988-5997, 2004.

4 Joensuu H, Kellokumpu-Lehtinen L, Bono P, Alanko T, Kataja V, Asola R, Utriainen T, Kokko R, Hemminki A, Tarkkanen M, Turpeenniemi-Hujanen T, Jyrkkio S, Flander M, Helle L, Ingalsuo S, Johansson K, Jaaskelainen S, Pajunen M, Rauhala M, Kaleva-Kerola J, Salminen T, Leinonen M, Elomaa I and Isola J: Adjuvant docetaxel or vinorelbine with or without trastuzumab for breast cancer. N Engl J Med *354*: 809-820, 2006.

5 Piccart-Gebhart MJ, Procter M, Leyland-Jones B, Goldhirsch A, Untch M, Smith I, Gianni L, Baselga J, Bell R, Jackisch C, Cameron D, Dowsett M, Barrios H, Steger G, Huang CS, Andersson M, Inbar M, Lichinitser M, Lang I, Nitz U, Iwata H, Thomssen C, Lohrisch C, Suter TM, Ruschoff J, Suto T, Greatorex V, Ward C, Straehle C, McFadden E, Dolci MS and Gelber RD: Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. N Engl J Med *353*: 1659-1672, 2005.

6 Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC and Ashworth A: Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature *434*: 917-921, 2005.

7 Dowsett M, Bartlett J, Ellis IO, Salter J, Hills M, Mallon E, Watters AD, Cooke T, Paish C, Wencyk PM and Pinder SE: Correlation between immunohistochemistry (HercepTest) and fluorescence *in situ* hybridization (FISH) for HER-2 in 426 breast carcinomas from 37 centres. J Pathol *199*: 418-423, 2003.

8 Baselga J, Carbonell X, Castaneda-Soto NJ, Clemens M, Green M, Harvey V, Morales S, Barton C and Ghahramani P: Phase II study of efficacy, safety, and pharmacokinetics of trastuzumab monotherapy administered on a 3-weekly schedule. J Clin Oncol *23*: 2162-2171, 2005.

9 Cox MC, Figg WD and Thurman PW: No rational theory for drug pricing. J Clin Oncol *22*: 962-963, 2004.

10 Elkin EB, Weinstein MC, Winer EP, Kuntz KM, Schnitt SJ and Weeks, JC: HER-2 testing and trastuzumab therapy for metastatic breast cancer: a cost-effectiveness analysis. J Clin Oncol *22*: 854-863, 2004.

11 Press MF, Sauter G, Bernstein L, Villalobos IE, Mirlacher M, Zhou JY, Wardeh R, Li YT, Guzman R, Ma Y, Sullivan-Halley J, Santiago A, Park JM, Riva A and Slamon DJ: Diagnostic evaluation of HER-2 as a molecular target: an assessment of accuracy and reproducibility of laboratory testing in large, prospective, randomized clinical trials. Clin Cancer Res *11*: 6598-6607, 2005.

12 Roche PC, Suman VJ, Jenkins RB, Davidson NE, Martino S, Kaufman PA, Addo FK, Murphy B, Ingle JN and Perez EA: Concordance between local and central laboratory HER2 testing in the breast intergroup trial N9831. J Natl Cancer Inst *94*: 855-857, 2002.

13 Paik S, Bryant J, Tan-Chiu E, Romond E, Hiller W, Park K, Brown A, Yothers G, Anderson S, Smith R, Wickerham DL, and Wolmark N: Real-world performance of HER2 testing–National Surgical Adjuvant Breast and Bowel Project experience. J Natl Cancer Inst *94*: 852-854, 2002.

14 Perez EA, Suman VJ, Davidson NE, Martino S, Kaufman PA, Lingle WL, Flynn PJ, Ingle JN, Visscher D and Jenkins RB: HER2 testing by local, central, and reference laboratories in specimens from the North Central Cancer Treatment Group N9831 intergroup adjuvant trial. J Clin Oncol *24*: 3032-3038, 2006.

15 Tubbs RR, Pettay JD, Roche PC, Stoler MH, Jenkins RB and Grogan TM: Discrepancies in clinical laboratory testing of eligibility for trastuzumab therapy: apparent immunohistochemical false-positives do not get the message. J Clin Oncol *19*: 2714-2721, 2001.

16 Jacobs TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Comparison of fluorescence *in situ* hybridization and immunohistochemistry for the evaluation of HER-2/*neu* in breast cancer. J Clin Oncol *17*: 1974-1982, 1999.

17 Fornier M, Risio M, Van Poznak C and Seidman A: HER2 testing and correlation with efficacy of trastuzumab therapy. Oncology (Williston Park) *16*: 1340-1348, 1351-1342; discussion *1352*: 1355-1348, 2002.

18 Tse C, Brault D, Gligorov J, Antoine M, Neumann R, Lotz JP and Capeau J: Evaluation of the quantitative analytical methods real-time PCR for HER-2 gene quantification and ELISA of serum HER-2 protein and comparison with fluorescence *in situ* hybridization and immunohistochemistry for determining HER-2 status in breast cancer patients. Clin Chem *51*: 1093-1101, 2005.

19 Jimenez RE, Wallis T, Tabasczka P and Visscher DW: Determination of Her-2/Neu status in breast carcinoma: comparative analysis of immunohistochemistry and fluorescent *in situ* hybridization. Mod Pathol *13*: 37-45, 2000.

20 Amr SS, Sa'di AR, Ilahi F and Sheikh SS: The spectrum of breast diseases in Saudi Arab females: A 26 year pathological survey at Dhahran Health Center. Ann Saudi Med *15*: 125-132, 1995.

21 Press MF, Bernstein L, Thomas PA, Meisner LF, Zhou JY, Ma Y, Hung G, Robinson RA, Harris C, El-Naggar A, Slamon DJ, Phillips RN, Ross JS, Wolman SR and Flom KJ: HER-2/*neu* gene amplification characterized by fluorescence *in situ* hybridization: poor prognosis in node-negative breast carcinomas. J Clin Oncol *15*: 2894-2904, 1997.

22 Ma Y, Lespagnard L, Durbecq V, Paesmans M, Desmedt C, Gomez-Galdon M, Veys I, Cardoso F, Sotiriou C, Di Leo A, Piccart MJ and Larsimont D: Polysomy 17 in HER-2/*neu* status elaboration in breast cancer: effect on daily practice. Clin Cancer Res *11*: 4393-4399, 2005.

23 Rasul KI, Mohammaed K, Abdalla AS, Chong-Lopez A, Ahmad MA, Al-Homsi MU, Al-Hassan AM, Al-Alosi AS and Bener A: Study of HER2/*neu* status in Qatari women with breast carcinoma. Saudi Med J *24*: 832-836, 2003.

24 Al-Kuraya K, Schraml P, Sheikh S, Amr S, Torhorst J, Tapia C, Novotny H, Spichtin H, Maurer R, Mirlacher M, Simon R and Sauter G: Predominance of high-grade pathway in breast cancer development of Middle East women. Mod Pathol *18*: 891-897, 2005.

25 Hoff ER, Tubbs RR, Myles JL and Procop GW: HER2/*neu* amplification in breast cancer: stratification by tumor type and grade. Am J Clin Pathol *117*: 916-921, 2002.

26 Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, Dowsett M, Fitzgibbons PL, Hanna WM, Langer A, McShane LM, Paik S, Pegram MD, Perez EA, Press MF, Rhodes A, Sturgeon C, Taube SE, Tubbs R, Vance GH, van de Vijver M, Wheeler TM and Hayes DF: 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, 2007.

27 Kallioniemi OP, Kallioniemi A, Kurisu W, Thor A, Chen LC, Smith HS, Waldman FM, Pinkel D and Gray JW: ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization. Proc Natl Acad Sci USA *89*: 5321-5325, 1992.

28 Masood S, Bui MM, Yung JF, Mark HF, Wong EY, Birkmeier JM, Yang SJ and Hsu P: Reproducibility of LSI HER-2/*neu* SpectrumOrange and CEP 17 SpectrumGreen Dual Color deoxyribonucleic acid probe kit. For enumeration of gene amplification in paraffin-embedded specimens: a multicenter clinical validation study. Ann Clin Lab Sci *28*: 215-223, 1998.

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

30 Jacobs TW, Gown AM, Yaziji H, Barnes MJ and Schnitt SJ: Specificity of HercepTest in determining HER-2/*neu* status of breast cancers using the United States Food and Drug Administration-approved scoring system. J Clin Oncol *17*: 1983-1987, 1999.

31 Bose S, Mohammed M, Shintaku P and Rao PN: Her-2/*neu* gene amplification in low to moderately expressing breast cancers: possible role of chromosome 17/Her-2/*neu* polysomy. Breast J *7*: 337-344, 2001.

32 Grushko TA, Blackwood MA, Schumm PL, Hagos FG, Adeyanju MO, Feldman MD, Sanders MO, Weber BL and Olopade OI: Molecular-cytogenetic analysis of HER-2/*neu* gene in BRCA1-associated breast cancers. Cancer Res *62*: 1481-1488, 2002.

33 Wang S, Saboorian MH, Frenkel E, Hynan L, Gokaslan ST and Ashfaq R: Laboratory assessment of the status of Her-2/*neu* protein and oncogene in breast cancer specimens: comparison of immunohistochemistry assay with fluorescence *in situ* hybridisation assays. J Clin Pathol *53*: 374-381, 2000.

34 Baselga J, Tripathy D, Mendelsohn J, Baughman S, Benz CC, Dantis L, Sklarin NT, Seidman AD, Hudis CA, Moore J, Rosen PP, Twaddell T, Henderson IC and Norton L: Phase II study of weekly intravenous recombinant humanized anti-p185HER2 monoclonal antibody in patients with HER2/*neu*-overexpressing metastatic breast cancer. J Clin Oncol *14*: 737-744, 1996.

35 Vogel CL., Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ and Press M: 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, 2002.

36 Slamon D and Pegram M: Rationale for trastuzumab (Herceptin) in adjuvant breast cancer trials. Semin Oncol *28(1 Suppl 3)*: 13-19, 2001.

37 Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S and Stewart SJ: First-line Herceptin monotherapy in metastatic breast cancer. Oncology *61(Suppl 2)*: 37-42, 2001.

38 Birner P, Oberhuber G, Stani J, Reithofer C, Samonigg H, Hausmaninger H, Kubista E, Kwasny W, Kandioler-Eckersberger D, Gnant M and Jakesz R: Evaluation of the United States Food and Drug Administration-approved scoring and test system of HER-2 protein expression in breast cancer. Clin Cancer Res *7*: 1669-1675, 2001.

Received August 3, 2010
Revised September 26, 2010
Accepted September 27, 2010