

RESEARCH COMMUNICATION

Efficacy of Primed In Situ Labelling in Determination of HER-2 Gene Amplification and CEN-17 Status in Breast Cancer Tissue

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Abstract

Considerable attention has been given to the accuracy of HER-2 testing and the correlation between the results of different testing methods. This interest reflects the growing importance of HER-2 status in the management of patients with breast cancer. In this study the detection of HER-2 gene and centromere 17 status was evaluated using dual-colour primed in situ labelling (PRINS) in comparison with fluorescence in situ hybridization (FISH). These two methods were evaluated on a series of 27 formalin fixed paraffin embedded breast carcinoma tumours, previously tested for protein overexpression by HercepTest (grouped into Hercep 1+/0, 2+ and 3+). HER-2 gene amplification (ratio \geq 2.2) by PRINS was found in 3:3, 6:21 and 0:3 in IHC 3+, 2+ and 1+/0 cases, respectively. Comparing FISH and IHC (immunohistochemistry), showed the same results as for PRINS and IHC. Chromosome 17 aneusomy was found in 10 of 21 IHC 2+ cases (47.6%), of which 1 (10%) showed hypodisomy (chromosome 17 copy number per cell \leq 1.75), 7 (70%) showed low polysomy (chromosome 17 copy number per cell=2.26 - 3.75) and 2 (20%) showed high polysomy (chromosome 17 copy number per cell \geq 3.76). The overall concordance of detection of HER-2 gene amplification by FISH and PRINS was 100% (27:27). Furthermore, both the level of HER-2 amplification and copy number of CEN17 analysis results correlated well between the two methods. In conclusion, PRINS is a reliable, reproducible technique and in our opinion can be used as an additional test to determine HER-2 status in breast tumours.

Keywords: PRINS - FISH - HER2/neu - aneusomy - breast cancer tissue

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Introduction

ERBB2/HER-2 (HER-2/neu, NEU, NGL, HER-2, TKR1, CD340) is a 185 kDa transmembrane growth factor receptor and one of the four members of type 1 growth factor receptor family, designated HER1 to HER4 (*c-erbB-1* to *c-erbB-4*). It has been shown to play a role in the signal transduction of cell growth but has no known natural ligand and instead seems to be activated via dimerisation with other receptors in the family: EGFR, HER3 or HER4 (Yarden and Sliwkowski, 2001). *HER-2* oncogene is located on the long arm of chromosome 17 (17q12-q21) (Owens et al., 2004) and plays a role in the pathogenesis of a significant number of human tumours. Approximately 20–30% of breast carcinomas and probably a higher percentage in the more malignant subgroups that form lymph node or distant metastases show altered HER-2 expression (Eccles, 2002; Carlsson et al., 2004). This is manifested as gene amplification and/or protein overexpression (Ross and Fletcher, 1999). It has been shown in many studies that overexpression of the HER-2 protein correlates with amplification of the HER-2 gene (Tubbs et al., 2000). These alterations are associated with shorter disease free period and overall

survival and with resistance to tamoxifen antiestrogen therapy and other chemotherapy regimens, regardless of the nodal or hormone receptor status (Tetu et al., 1998). Moreover, patients suffering breast carcinoma presenting HER-2 amplification or overexpression can benefit from anthracycline-based regimens, as well as trastuzumab (Cobleigh et al., 1999).

On the other statement HER-2 status in breast cancer is used as a prognostic factor, a predictive factor, and a therapy selection factor (Wolff et al., 2007) for the humanized monoclonal antibody trastuzumab (Herceptin®; Genentech), which is an FDA approved drug for use as monotherapy or combined chemotherapy for treatment of breast cancer patients with amplified HER-2 status. Trastuzumab adjuvant treatment for early HER-2 positive breast cancer is effective for improving patient survival and cost-effectiveness analyses of such treatment have shown acceptable ratios (Fagnani et al., 2007; Garrison et al., 2007; Millar and Millward, 2007; Norum et al., 2007). However, there is a negative aspect to trastuzumab therapy, namely cardiac toxicity (Wolff et al., 2007), which is possibly due to myocardial HER-2 gene over-expression associated with anthracycline treatment (De Korte et al., 2007) and substantial trastuzumab therapy

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costs.

The American Society of Clinical Oncology (ASCO) published an update of recommendations for use of HER-2 as a biomarker for breast cancer patients (Harris et al., 2007). According to this updated guideline, HER-2 should be evaluated in every primary invasive breast cancer either at the time of diagnosis or at recurrence in order to guide selection of trastuzumab for treatment. Some other recommendations were also made regarding utility of HER-2 assessment to predict sensitivity to specific chemotherapeutic agents (Shah and Chen, 2010).

Detection technologies have emerged as important components of healthcare and are increasingly used for cancer therapies. Since significant contradictions in various studies can in part be attributed to differences in HER-2 testing and interpretation (Hanna et al., 1999), it is critical to validate and standardize these techniques in order to make an accurate assessment of HER-2 status. A critical challenge to the implementation of targeted cancer therapies is the determination of whether and how they will be provided to the individuals who will benefit most from them. HER-2 testing to target trastuzumab treatment for patients with breast cancer is a well-known example of the successful use of testing to target cancer treatment that has been used in clinical practice for over 10 years (Phillips et al., 2009).

Techniques which have been used to assess HER-2 protein overexpression are immunohistochemistry, ELISA analysis of tumor cytosols or serum, and Western blot, and methods used to evaluate HER-2 gene amplification include Southern blot, slot blot, CISH, FISH, and PCR (Ross et al., 2009). Blotting methods (such as Southern, Northern, and Western) used to measure HER-2 molecules are technically difficult, require large amounts of fresh tissue, and are impractical for routine screening purposes. In addition, these techniques are not tumor cell-specific. Thus, the HER-2 status would be modified by the dilutional effect caused by the large numbers of non-neoplastic cells (inflammatory, stromal, and normal) found in all tumours, resulting in an underestimation of gene amplification or expression. This problem can be reduced if analysates are enriched for target cells of interest (Gjerdrum et al., 2001). PCR is a sensitive technique; however, it is also affected by dilutional artifacts, and the analysis is time consuming and labour intensive. The absence of simultaneous morphological assessment in the above studies is also a significant disadvantage.

Contrary to the above, analysis by IHC and FISH, the most commonly used assays in the clinical setting for evaluating HER-2 status, approved by FDA, can be automated and allow the simultaneous assessment of tumor morphology while eliminating difficulties with dilution artifacts. However, the HER-2 -IHC detection was criticised because of a lack of interlaboratory reproducibility and, furthermore, Herceptests, a standardised IHC method, was shown to be a method with excessive sensitivity when compared to FISH (Tubbs et al., 2001).

FISH identifies the number of copies of the HER-2 gene, normally in conjunction with the number of chromosome 17 centromere copies, and is generally seen

as being more quantitative than IHC. Furthermore, as DNA is more stable than protein, pre-analytical factors have less impact on test results compared with IHC. However, it is more expensive than IHC and takes longer to perform. It also requires expert technicians and access to a fluorescence microscope. Also the signals produced by the FISH assay decay within a few weeks (Van de Vijver et al., 2007). More recently, the CISH (chromogenic in situ hybridization) methodology, approved by FDA, has emerged as a potential alternative to FISH (Rosa et al. 2009). Other new modalities of HER-2 testing are Metallographic In Situ Hybridization (Tubbs et al., 2002; Downs-Kelly et al., 2005) and brightfield double in situ hybridization (Nitta et al., 2008).

Current recommendations of the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) include determination of HER-2 status in all invasive breast cancers using IHC or ISH (García-Caballero et al., 2010). The guidelines by ASCO/CAP define an HER-2 IHC staining of 3+ as uniform intense membrane staining in >30% of invasive tumor cells as compared to previously defined >10% strong staining. Cases with weak to moderate complete membrane staining in at least 10% of cells are considered equivocal (2+), and in these cases, HER-2 gene amplification with fluorescent in situ hybridization (FISH) should be tested. For FISH, the tumor is negative for HER-2 gene amplification if the ratio of HER-2 gene signals to chromosome 17 signals is <1.8 or HER-2 gene copy number is <4.0, equivocal when the ratio is 1.8–2.2 or HER-2 gene copy number is 4.0–6.0 and positive if the ratio is >2.2 or HER-2 gene copy number is >6.0 (Shah and Chen, 2010).

The primed in situ labelling, a technique called PRINS; which is a combination of FISH and in situ polymerisation, provides another approach for in situ chromosomal detection. In this procedure, introduced by Koch et al. (1989) the chromosomal identification is performed by in situ annealing of specific and unlabeled oligonucleotide primers to complementary sites on denatured chromosome spreads, nuclei or tissue sections. Cells or tissue samples are fixed and denatured before PRINS reaction, both to preserve morphology and to permit access of the reagents to the sequence target. The annealed primers provide initiation sites for chain elongation catalyzed by a Taq DNA polymerase in the presence of free nucleotides, of which at least one is labelled. The in situ visualization of generated fragments results from the incorporation of the labelled nucleotide (Pellestor, 1998).

Because of its relative simplicity and the commercial availability of numerous DNA probes, fluorescence in situ hybridization has become the standard technique for in situ chromosomal investigations. However, the PRINS reaction offers a fast alternative approach based on the use of short, unlabeled, and chromosome-specific primers (Koch et al., 1989). The lengths of the PRINS primers range from 18 to 30 nucleotides. Compared to the size of DNA repetitive probes (250–600 bp), this small size greatly facilitates their in situ accessibility to the genomic target sequences. This is particularly significant in cells with highly condensed nuclei. Because they are unlabeled, high amounts of primers can also be used in

PRINS reaction without inducing background signals. The complementation process between the primer and its target will be so specific that a simple mismatch between the 3'-end of the primer and the genomic sequence will prevent initiation of the in situ elongation by the Taq DNA polymerase (Pellestor, 2006). Based on the use of such primers, the PRINS reaction combines the high sensitivity of polymerase chain reaction (PCR) with the cytological localization of DNA sequences (Koch et al., 1989).

Thus, semi automatic PRINS protocols have been developed offering a high reproducibility in labelling reaction. An additional improvement was the direct use of fluorochromes in sequential PRINS reactions. A multicolour PRINS protocol has been reported, allowing performance of ultra-rapid detection on several chromosomes, by mixing the different fluorochromes during the chain elongation reaction (Yan et al., 2001). PRINS reactions are fast, and the resulting data can be obtained in less than 4 hrs, whereas FISH results for HER-2 detection are generally obtained at least after 16-20 hrs or even more.

In humans the PRINS method has been successfully tested for the assessment of aneuploidy in lymphocytes, spermatozoa, oocytes, amniocytes and preimplantation embryos (Speel et al., 1995; Pellestor et al., 1996; Mennicke et al., 2003; Pellestor, 2006). The use of PRINS has also been reported for analysis of structural aberrations such as translocations and marker chromosomes and localization of single copy genes such as SRY and SOX3 (Kadandale et al., 2000a, 2000b) as well as for the detection of fetal cells in peripheral venous blood of pregnant women (Orsetti et al., 1998; Krabchi et al., 2001; Krabchi et al., 2006). Further applications of PRINS have also been reported for tumoral cytogenetics (Tharapel and Kadandale, 2002).

In the present study, the utility and efficiency of the PRINS method was investigated in the detection of HER-2 gene amplification and CEN-17 status in FFPE tumor breast tissues.

Materials and Methods

Study design and tumour specimens

Routine formalin-fixed paraffin-embedded breast cancer specimens from 27 patients were included in this study. Specimens were previously formalin-fixed (fixation time ranged from 12–48 hours) and embedded in paraffin blocks. The tissues were sectioned with 4–5 μ m thickness, mounted on coated (poly L-lysine) slides and baked overnight in 56–60 °C. All specimens were coded for the study. The study was approved by the Ethical Committee of the Faculty of Medical Sciences of the Tarbiat Modares University (Tehran, Iran). Specimens were selected based on their HercepTest immunohistochemical (IHC) score, so that three of them were HER-2 negative (0/1+), three were HER-2 positive (3+) and 21 were equivocal (2+) based on IHC score. PRINS and FISH were used to assess HER-2 gene amplification and CEN-17 status in all specimens.

Fluorescence in situ hybridization (FISH)

All the solutions and reagents used in this research

were purchased from MERCK except the ones specified. Kreatech Poseidon TM Repeat-FreeTM fluorescent labelled DNA probes (Kreatech Diagnostic, Netherlands) were used and the specimen preparation, hybridization and post-hybridization washes were performed as per the manufacturer's suggestions with slightly modifications. Briefly, after deparaffinizing the unstained sections in xylene for 10 minutes three times, the sections were rehydrated in 100%, 85% and 70% ethanol for 2 minutes each and air-dried. Subsequently, the sections were immersed in 0.2N HCl for 20 minutes in room temperature, rinsed in purified water for 3 minutes and washed in 2 \times SSC for 3 minutes. Sections were then treated with sodium thiocyanate solution 1N at 80°C for 10 minutes followed by rinsing in purified water for 1 minute and washing in 2 \times SSC for 5 minutes twice. The sections were then subjected to protease digestion [Pepsin (Sigma USA) 4 mg/ml in 0.2N HCl] at 37 °C for 10 minutes, washed in distilled water and air-dried. dehydrated in grades of alcohol (1 minutes each in 70%, 85% and 100%). Hybridization with POSEIDON Repeat-Free (Kreatech) probes was carried out overnight. Next morning, the sections were washed in 0.4 \times SSC / 0.3% igeal for 2 min at 72 °C (\pm 1), followed by 2 \times SSC/0.1% igeal for 1 minutes at room temperature. The sections were counterstained with DAPI antifade (Cytocell, U.K). All slides were analyzed using a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) equipped with epifluorescence and triple band pass filter.

Primed in situ labelling (PRINS)

The pre-treatment steps are the same with steps described in FISH procedure. After protease digestion followed by washing and de hydration, PRINS procedure continued as follows. The reaction mixture for each PRINS primer (40 μ l) consisted of: 0.2 mM of each of dATP, dCTP and dGTP, 0.02 mM of dTTP (Roche, Germany) 0.5 μ l of tetramethyl-rhodamine-5- dUTP for HER-2 or fluorescein-12-dUTP for CEN-17 detection (Roche diagnosis, Germany), 50 pM of each primers (table 1) 0.5 μ l of Taq DNA polymerase, 5 μ l Taq polymerase buffer with MgCl₂, 0.01% of bovine serum albumin (Clontech, Inc., Palo Alto, CA) and distilled water. The reaction was performed on a programmable Eppendorf thermocycler fitted with a flat plate block. After application of PRINS mixture for HER-2 onto the slides they were put on the plate block. The first heating step (3 min at 94°C) for DNA denaturation followed by 10 min. in proper annealing temperature (60°C for HER-2) allowed the primer to anneal. The temperature was then automatically raised to 72°C for the 15 min elongation step. Then cover slip was removed and the first PRINS reaction was arrested by immersing the slides in a stop solution (500mM NaCl/50 mM EDTA, pH 8) at 72°C for 3 min. The slides were then transferred from the stop solution to 1 \times NT buffer (50 mM Tris-HCl, pH 7.2; 10 mM MgSO₄; 10 mM dithiothreitol, 0.15mg/mL BSA) and washed twice for 5 min each at room temperature before being treated with 40 μ L dideoxynucleotides mix (10 μ M each of ddATP, ddCTP, ddGTP, ddTTP, 4 μ l 10 \times NT buffer, and 1 U of Klenow enzyme) (Roche, Germany) for 10 min at 37 °C in

order to block the free 3' ends of the elongation fragments generated by the first PRINS reaction. This intermediate step prevented mixing of labelling. The slides were then passed in stop solution and washed twice in 1 × NT buffer at room temperature. The second PRINS reaction mixture containing CEN-17 primer and fluorescein-12-dUTP was applied on the slide, and covered with a 22×32 cover slip. Then the slide was placed again on the plate of the thermal cyclor.

The program used for the second PRINS reaction was: 5 min at the annealing temperature (50°C), specific to the second primer used followed by 10 min in extension temperature (72 °C). No additional denaturation was required after the first PRINS reaction because DNA remains denatured through the PRINS incubations. Upon completion of the program, the slides were washed in 4 × SSC / 0.05% Tween 20 for 5 min at room temperature the slides were counterstained with DAPI-antifade (Cyclocell, UK). All slides were analyzed using a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan) equipped with epifluorescence and triple band pass filters.

Scoring of the PRINS and FISH slides

For all the tumour specimens the HER-2 and centromere 17 (CEN-17) signals from 50-100 nuclei were counted and the FISH and PRINS scores were expressed as ratio of HER-2 signals (spectrum orange) per chromosome 17 signals (spectrum green). If the ratio of spectrum orange to spectrum green was ≥ 2.2, then the sample was considered to have HER-2 gene amplification (cut off value for HER-2 amplification) and if the ratio was 0.8 > x < 2.2 or ≤ 0.8 then the sample was considered to have no HER-2 gene amplification or to have HER-2 gene deletion, respectively. The slides were first scanned

Table 1. Characteristics of Designed Oligonucleotides Used as PRINS Primers

Gene name	Primer sequence (5'-3')
HER-2 (primer1)	CCTCTGACGTCCATCATCTC
HER-2 (primer2)	ATATCCTCCTCTTTCTGCC
HER-2 (primer3)	CTGGTACTTTGAGCCTTAC
HER-2 (primer4)	CATTCCAGGGGATGAGCTA
HER-2 (primer5)	CTACCTGCCATGATGCTAGA
CEN 17	AATTTACAGCTGACTAAACA

* HER-2 Primers were designed using Primer express software version 3.0 and ordered to synthesis to Bionneer company (USA). CEN 17 primer previously used by Coullin et al. 1997.

Table 2. Cross-tabulation of HER-2 Status Based on HER-2/CEN-17 Ratio for Fluorescence in Situ Hybridization (FISH) and Primed In Situ labelling (PRINS)

PRINS HER2 status	FISH HER2 status			total
	Normal	deleted	amplified	
Normal	17	0	0	17
deleted	0	1	0	1
amplified	0	0	9	9
Total	17	1	9	27

* Agreement 100.0%, κ value 1.00

at 100x magnification to ensure objective evaluation of signals. Precise signal enumeration of high level amplification (>30× ratio) was not possible because of coalescing fluorescence of signal clusters. Aneusomies of CEN-17 assessed in this study contained hypodisomy (chromosome 17 copy number per cell ≤ 1.75), low polysomy (chromosome 17 copy number per cell=2.26 approximately 3.75) and high polysomy (chromosome 17 copy number per cell ≥ 3.76).

Statistical analyses

Following scoring by dual-colour PRINS and FISH, the data were combined. The correlation between dual-colour PRINS and FISH results with respect to both gene copy number and ratios for HER-2 and CEN-17 were analysed and the correlation coefficients calculated. Concordance between HER-2 status in the dual-colour PRINS, FISH and IHC assays was evaluated by calculating the percent agreement and by κ statistics (Koch et al., 1977). All statistical analyses were performed using SPSS 16 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Twenty seven formalin fixed paraffin embedded breast cancer tumor slides with known IHC status, were assessed to determine HER-2 amplification status by PRINS and FISH techniques. The HER-2 status of normal or amplified was assigned to all breast cancer slides based on the HER-2:CEN-17 ratio determined in both the dual-colour PRINS and FISH protocols. Specimens with a HER-2:CEN-17 ratio 0.8 > x < 2.2 were scored as normal, whereas those with a HER-2:CEN-17 ratio ≥ 2.2 were scored as amplified and HER-2:CEN-17 ratio ≤ 0.8 considered to have HER-2

Table 3. Cross-tabulation of HER-2 Status for Immunohistochemistry (IHC)

PRINS HER2 status	HER2 IHC status		
	negative	positive	total
Dual-colour Primed In Situ labelling (PRINS) without IHC 2+ cases			
Normal	3	0	3
amplified	0	3	3
Total	3	3	6
Fluorescence in situ hybridization (FISH) without IHC 2+ cases			
Normal	3	0	3
amplified	0	3	3
Total	3	3	6

* Agreement 100.0%, κ value 1.00

Table 4. Cross-tabulation of Aneusomy CEN-17 Status for Fluorescence in Situ Hybridization (FISH) and Primed In Situ labelling (PRINS) in IHC 2+ cases

PRINS CEN-17 aneusomy status	HER2 IHC status			
	Hypodisomy	Low*	High*	total
Hypodisomy	1	0	0	1
Low polysomy	0	7	0	7
High polysomy	0	0	2	2
Total	17	2	1	0

* polysomy Agreement 100.0%, κ value 1.00,

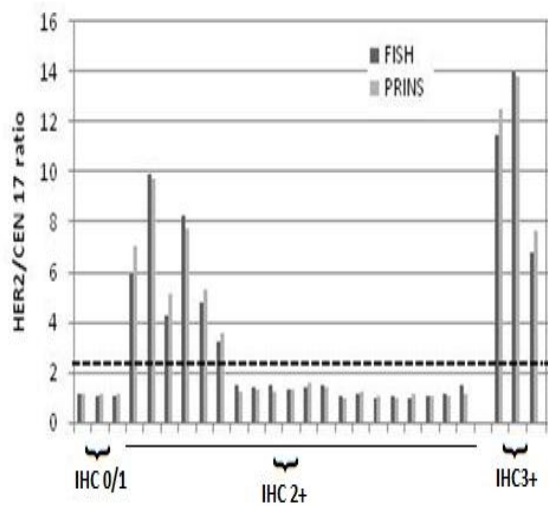


Figure 1. Individual Paired HER-2 :CEN-17 Ratios for dual-colour Primed In Situ labelling and Fluorescence in Situ Hybridization. Data are correspond to 27 individual cases ($n = 27$) in cases classified by the Hercep-Test protocol in each of the four immunohistochemical categories 0+/1+ (negative), 2+ (equivocal) or 3+(positive). The dashed line illustrates the cut-off value of 2.2

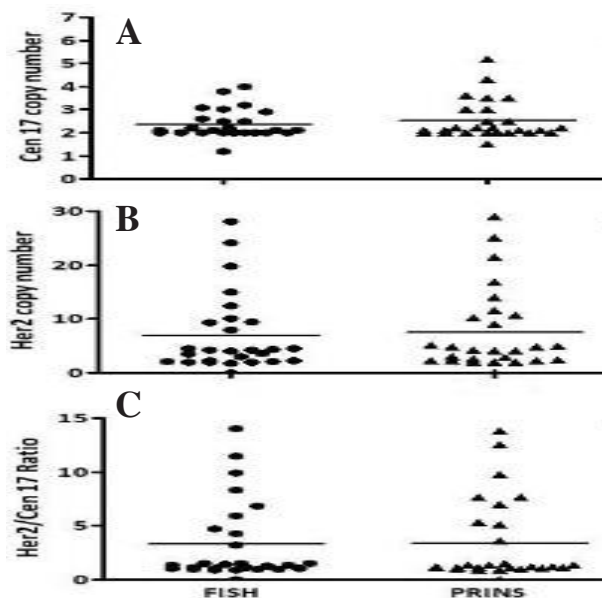


Figure 2. A) Cen-17 Copy Numbers Determined by Dual Colour PRINS and FISH. B) HER-2 Copy Numbers Determined by Dual Colour PRINS and FISH. C) HER-2/Cen-17 Ratios Determined by Dual Colour PRINS and FISH

gene deletion. All the three IHC negative cases did not show HER-2 gene amplification whereas the three IHC positive cases showed HER-2 gene amplification using both FISH and dual-color PRINS methods. From 21 IHC 2+ cases, 6 cases showed HER-2 gene amplification, 1 HER-2 gene deletion and the rest (14 cases) showed no HER-2 gene amplification when assessed by FISH and PRINS. The agreement between HER-2 status when determined by dual-colour PRINS and FISH analysis was found to be 100.0% (χ value=1.00), corresponding to perfect agreement between these two methods (Table 2).

Furthermore, to enable comparisons of dual-colour

PRINS and FISH with the HercepTest IHC score, scores of 0 and 1+ were regarded as negative (normal), whereas a score of 3+ was regarded as positive (amplified). When comparing the IHC HER-2 status, without the equivocal IHC2+ cases, with the status obtained in the dual-colour PRINS or FISH protocols, 100% agreement (χ value = 1.00) was observed for IHC versus dual-colour PRINS (Table 3) and for IHC versus FISH (Table 4).

In Figure 1 individual paired HER-2 : CEN-17 ratios determined by the dual-colour PRINS and FISH protocols have been graphed. Good agreement between the ratios determined by the two methods is obtained.

In Figure 2, the agreement between the dual-colour PRINS and FISH methods has been shown by plotting the HER-2 :CEN-17 ratios, the HER-2 copy numbers and the CEN-17 copy numbers found by the two methods. Comparison of the HER-2:CEN-17 ratios found in the dual-colour PRINS and FISH analyses by a paired t-test revealed no significant difference between these ratios. Furthermore, we observed that the mean dual-colour PRINS HER-2 and CEN-17 copy number was not significantly different from the corresponding mean FISH HER-2 and CEN-17 copy number in a paired t-test.

Analysis of the cases with aneusomy of centromere 17 showed that 10:21(47.6%) of IHC 2+ had aneusomy in their CEN-17 of which 1:10 (10%) were hypodisomy (CEN-17 mean copy number < 1.75), 7 :10 (70%) were low- polysomy (CEN-17 mean copy number 2.26-3.75) and 2:10 (20%) were high -polysomy (CEN-17 mean copy number > 3.75). The concordance between dual-colour PRINS and FISH methods were 100% (χ value = 1.00), corresponding to perfect agreement between these two methods.

Discussion

Accurate HER-2 status testing is important for identifying breast cancer patients who may benefit from receiving trastuzumab therapy. Moreover, in the future, HER-2 status may also help select patients for tyrosine kinase inhibitor therapy (Moasser et al., 2001). Currently, in the United States, HER-2 IHC method is most commonly used technique for primary screening of HER-2 status, and borderline cases are subjected to dual FISH for HER-2 and CEN 17 to determine the HER-2/CEN 17 ratio. Because the discordance rate between local and central/reference HER-2 status testing with IHC and FISH is significantly high (Perez et al., 2002; Dowsett et al., 2007) the standardization of diagnosing breast cancer cases is recognized as a very important task for improving personalized cancer patient care (Wolff AC, et al., 2007; Ross et al., 2007).

Several reasons could account for the low sensitivity with the IHC assay. Since the assay is directed towards the detection of protein, the technical considerations such as pre-analytical tissue processing, reagent variability, antigen retrieval and very subjective scoring might adversely affect the result. A FISH assay directed at the gene itself might overcome all these drawbacks. FISH has several advantages over IHC such as ease of use, reproducibility and very objective scoring criteria. As shown by several studies, FISH is highly reproducible and reliable with very limited reagent variation (Ganberg

et al., 2002; Gupta et al., 2003), but is time consuming and requires special training and access to a fluorescence microscope also the signals produced by the FISH assay decay within a few weeks. CISH is an alternative method to evaluate amplifications that requires a conventional light microscopy, permits a more rapid interpretation time and a review of the morphological details. A further advantage of CISH is that the probe signals are permanent and the slides can therefore be archived for long periods of time (Kim and Oh, 2004).

A key advantage shared by IHC, CISH and FISH techniques for HER-2 evaluation is that they are based on microscopic analysis. In principle, this allows for changes in gene copy number and gene product expression to be assessed specifically in the tumor cell population. Most in situ hybridization protocols are quite complicated and time consuming. They require sophisticated pretreatments that have to be optimized according to the probe and tissue used with frequent overnight incubation.

The PRINS technique is much more simple, efficient, and faster. Since the oligoprobe is unlabeled and labelling occurs only secondarily to specific hybridization, the background staining is minimal. The use of an inexpensive unlabeled probe makes it possible to use high probe concentrations and short reaction times. The increase in signal intensity is most prominent when oligoprobes are used because the chain elongation is independent of the length of the primer (Koch, 1992).

The protocol described in this paper can be completed in less than 3 hours. Detection of HER-2 gene amplification on paraffin-embedded material with conventional in situ hybridization takes several hours to overnight hybridization, resulting in lengthy procedures. Because of the high complementarity between the oligonucleotide primer and its genomic target, PRINS appears to be more efficient than FISH for discriminating α -satellite DNA sequences. In a PRINS reaction, a single mismatching base pair at the 3' end of the oligonucleotide primer may prevent the in situ elongation by Taq polymerase (Bottema and Sommer, 1993). In FISH reaction, the stability of the probe-target hybrid is not affected by such mistakes.

As well as fluorescent dyes, visualization of the PRINS reaction product is possible with an enzymatic method (alkaline phosphatase), thereby omitting the need for an ultraviolet microscope to evaluate the results. This results in permanent preparations can be easily studied by a classical light optical microscope (Herrington et al., 1990). In an era of patient-specific therapy, the clinical importance of demonstrating HER-2/neu amplification and/or high gene expression is compelling. What is less clear, is the optimal method for evaluating HER-2 status in the routine clinical setting. In the present study, we showed that by combining the precision and high sensitivity of PCR analysis with the cytological localization of DNA sequences, using PRINS technique, it was possible to quantify both HER-2 gene amplification and CEN-17 status in routine FFPE archive tissues.

A consensus panel has proposed adapted scoring guidelines for HER-2 testing. An important recommendation from this panel was to consider reporting breast cancer cases with a HER-2/centromere chromosome 17 ratio between 1.8 and 2.2 as borderline (Dowsett et al., 2007). Using this adapted scoring

guideline, a tumour is assessed as HER-2 amplified when the ratio is more than 2.2; or when the absolute number of HER-2 gene copies is more than six.

Our results showed a very good concordance between FISH and dual colour PRINS in all the experienced groups with known IHC status. This analysis has revealed a significant correlation of copy numbers for HER-2, CEN-17 and the HER-2 : CEN-17 ratio between the dual-colour PRINS and FISH protocols. In both control groups, IHC positive and negative ones, 100% agreement was shown between IHC, FISH and dual-color PRINS methods. In the IHC equivocal group (IHC 2+), 6:21 (28.57%) of cases showed HER-2 gene amplification. In other studies HER-2 gene amplification in IHC 2+ status cases were reported 27.91% in Iranian populations (Ghaffari et al., 2011) and a range from 20% to 76.19% in the other populations (20% (Mrozkowiak et al., 2004), 23% (Rasmussen et al., 2008; Al-Khattabi et al., 2010), 24% (Dybdal et al., 2005), 28.1% (Park et al., 2011), 30.0% (Singhai et al., 2011), 32% (Ellis et al., 2005), 33.3 (Moerland et al., 2006), 37% (Perez et al., 2002), 66.6% (Panjwani et al., 2010), 76.19 (Zeng et al., 2006)).

It was found one out of 21 cases showed deletion of HER-2 gene in IHC equivocal group using dual-colour PRINS. This finding was also confirmed by FISH method. The other study had shown HER-2 gene and/or CEN-17 deletion in 2% of IHC 2+ cases (Perez et al., 2002). Polysomy of chromosome 17 is frequent, and depending on the definition of polysomy, it may be seen in 20%–30% of invasive breast carcinomas (Downs-Kelly et al., 2005; Torrisi et al., 2007; Hyun et al., 2008; Shah et al., 2009). Analysis of polysomy 17 requires the use of dual colour FISH, and its presence can complicate accurate assessment of HER-2 status (Wang et al., 2002). Studies have shown polysomy 17 as a contributing factor in a small subset of tumours, which were IHC3+ but lacked HER-2 gene amplification (Lal et al., 2003; Hyun et al., 2008; Shah et al., 2009). A recent analysis of HER-2 status by array comparative genomic hybridization in breast carcinoma samples (n = 97) has shown that polysomy 17 is a rare event and suggest that the cases detected by FISH represent amplification of chromosome 17 centromere rather than true polysomy (Yeh et al., 2009).

As well as double-colour FISH analyses, dual-colour PRINS may give more information, particularly the ratio between HER-2 signal and the number of chromosome 17, and may separate the high polysomy of chromosome 17 and the very low level of HER-2 amplification. It has been stated that polysomy of chromosome 17 is statically more frequent in IHC 2+ tumours. In routine, these situations are very infrequent and it is not proved that this distinction is relevant in terms of response to Herceptin therapy. Clinical trials, including a large number of IHC 2+ tumours with a low level of amplification, are needed to confirm that the exact level of HER-2 gene amplification is important for the patient's selection for specific therapy (Arnould et al., 2003).

Our results showed that about 47.6% (10:21) IHC 2+ cases, showed CEN-17 aneusomy when analysed by dual-colour PRINS and FISH. These aneusomies were consisted of 10% (1:10) hypodisomy, 70% (7:10) low polysomy and 20% (2:10) high polysomy. This finding was somehow in line with other studies showed 19.5%

CEN-17 polysomy (Vanden Bempt et al., 2008) and 59.52% CEN-17 aneusomy (Zeng et al., 2006) of IHC 2+ cases. We here report a 100% concordance in HER-2 status between dual-colour PRINS and FISH as well as a great reduction in procedure time and costs when using the dual-colour PRINS protocol compared with FISH.

In conclusion, in our opinion, PRINS is too sophisticated to be an alternative to IHC screening of all the breast tumours. However, because of the good correlation between PRINS and FISH, even in ambiguous IHC results, this finding opens the possibility for PRINS to be used for the determination of gene amplification status in IHC 2+ tumours.

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