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HER-2 DNA quantification of paraffin-embedded breast carcinomas with LightCycler real-time PCR in comparison to immunohistochemistry and chromogenic in situ hybridization

Maria Ntoulia [a], Loukas Kaklamanis [b], Christos Valavanis [c], Maria Kafousi [d], Efstathios Stathopoulos [d], Petroula Arapantoni [c], Dimitris Mavroudis [e], Vassilis Georgoulias [e], Evi S. Lianidou [a,*]

[a] *Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Athens, 15771, Greece*
[b] *Department of Pathology, Onassis Cardiac Surgery Center, Athens, 17674, Greece*
[c] *Molecular Pathology Unit, Department of Pathology, Metaxas Memorial Cancer Hospital of Piraeus, Greece*
[d] *Department of Pathology, University General Hospital of Heraklion, Crete, 71110, Greece*
[e] *Department of Medical Oncology, University General Hospital of Heraklion, Crete, 71110, Greece*

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Abstract

Objectives: To compare the detection of *HER-2* status by real-time PCR, on paraffin-embedded breast carcinomas, in respect to immunohistochemistry (IHC) and chromogenic in situ hybridization (CISH).

Design and methods: Paraffin-embedded breast carcinomas collected from 85 patients diagnosed with early stage breast cancer were analyzed for *HER-2* gene amplification by real-time PCR and CISH, as well as for *HER-2* protein expression by IHC.

Results: *HER-2* gene amplification was observed in 19 (22.4%) of 85 breast cancer patients by real-time PCR and in 19 (22.4%) of 85 patients by CISH. Strong (3+) *HER-2* protein over-expression was observed in 13 (15.3%) out of 85 patients. Moreover, there were 4 out of 85 (4.7%) patients that had moderate (2+) *HER-2* protein over-expression, while 68 out of 85 (80%) patients had no *HER-2* protein over-expression by IHC. There were strong concordance rates between real-time PCR and IHC (79/85, 92.9%, $p<0.0001$) and real-time PCR and CISH (77/85, 90.6%, $p<0.0001$). The concordance rate between the three methods was 90.6% ($p<0.0001$).

Conclusions: Our data show that the results obtained for amplification of *HER-2* by real-time PCR on the LightCycler are comparable to those obtained by IHC and CISH.

Keywords: *HER-2* gene amplification; Breast carcinomas; Real-time PCR; CISH; IHC

Introduction

The epidermal growth factor (EGF) family consists of four receptors, HER-1, HER-2, HER-3 and HER-4, with considerable homology. Upon ligand binding, the receptors homo-dimerize and hetero-dimerize generating different biological signals [1]. The epidermal growth factor system stimulates cell proliferation, prevents apoptosis and stimulates angiogenesis and metastasis via EGFR-related pathways. The *HER-2* gene, also known as c-erbB-2 or HER2/neu, encodes a 185 kDa transmembrane protein with tyrosine kinase activity, which participates in the regulation of cell growth [1]. Twenty to 30% of invasive breast carcinomas show *HER-2* over-expression as a result of gene amplification. Over-expression of *HER-2* is associated with an aggressive tumor phenotype that is characterized by relatively rapid tumor growth, metastatic spread to visceral sites and drug resistance. Targeted blockade of *HER-2* with trastuzumab (Herceptin) has been shown to improve survival in women with *HER-2* positive advanced breast cancer [2].

Knowledge of *HER-2* status is a prerequisite when considering a patient's eligibility for trastuzumab therapy. Recently, the

* Corresponding author. Fax: +30 210 7274750.
E-mail address: lianidou@chem.uoa.gr (E.S. Lianidou).

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testing guidelines implemented by many countries in order to standardize testing procedures for *HER-2* analysis have been reviewed [3]. *HER-2* gene amplification may be determined by real-time PCR, fluorescence in situ hybridization (FISH) or chromogenic in situ hybridization (CISH) whereas *HER-2* over-expression at the protein level may be identified by immunohistochemistry (IHC) [1]. IHC is widely used since it is semi-quantitative, faster and economical than FISH; however it is highly dependent on the specificity of Abs used and is difficult to be standardized, because of pre-analytical variables, antibody sensitivity and specificity and inter-observer variability [4–6].

The FISH assay for *HER-2* status is technically more reproducible than IHC, enabling the quantification of *HER-2* gene copy number in each tumor cell and is considered as the gold standard method for detecting *HER-2* gene amplification [7]; however, CISH has emerged as a potential alternative to FISH, since CISH signals are more permanent, and the method is less expensive and is more practical for the evaluation of *HER-2* gene status [8,9]. Several studies have demonstrated high concordance between FISH and CISH [10,11], as well as between IHC and FISH or CISH [12–17].

Recently developed PCR-based assays can determine changes in both *HER-2* gene copy number and expression [18–20]. Quantitative real-time PCR is a relatively new and alternative technique for assessing *HER-2* gene amplification. In this methodology, PCR amplification and quantification are performed in the same reaction tube thereby eliminating, several technical problems and methodological inconveniences. The high sensitivity of real-time PCR means that even minute amounts of DNA or RNA may be detectable in formalin-fixed paraffin-embedded tissues opening up the possibility of performing retrospective clinical and molecular studies on archival tissue stored in pathology institutes [21,22]. So far, several studies have compared *HER-2* DNA amplification by real-time PCR with FISH and IHC. Most of these studies show a good correlation between real-time PCR and the other two methods [19–24]. However, so far there are no data comparing the effectiveness of real-time PCR in respect to CISH for assessing *HER-2* status.

In the present study, we report, for the first time, the comparison of *HER-2* status detection by real-time PCR in 85 archival formalin-fixed paraffin embedded tissues from patients with operable breast cancer, in comparison to CISH and IHC.

Methods

Tissue samples

The study material consisted of paraffin-embedded breast carcinomas, obtained from 85 patients with early stage breast cancer followed at the Department of Medical Oncology, University General Hospital of Heraklion, Crete. All patients gave their informed consent and the study has been approved by the Ethical and Scientific Committees of our Institution. A representative block of paraffin-embedded tumor tissue from each patient was selected and used to prepare 5 μm sections for IHC and CISH. Two additional sequential 10 μm sections were taken from the same block for DNA extraction and real-time PCR. To minimize dilution of the PCR signal by non tumoral and non-amplified cells, sections containing >80% tumor cells were selected for DNA extraction. DNA was extracted from these sections with the High Pure PCR Template Preparation kit (Roche, Penzberg, Germany) according to the manufacturer's protocol [23].

Real-time PCR

Real-time PCR was performed in the LightCycler (Roche, Penzberg, Germany) and the "LightCycler-*HER-2*/neu DNA quantification kit" (Roche, Penzberg, Germany) was used for *HER-2* gene copy quantification according to the manufacturer's instruction [23]. For each reaction, 2 μL Light-Cycler-*HER-2*/neu detection mix, 2 μL Light-Cycler-HER2/neu reference gene detection mix, 2 μL LightCycler-*HER-2*/neu enzyme master mix and 9 μL PCR grade water supplied with the kit were combined and aliquoted in the capillaries; 5 μL of either human DNA extracted from the tumor tissue or "Light-Cycler-HER2/neu calibrator DNA" or PCR grade water (as a negative control) was added to give a total volume of 20 μL. PCR was performed as follows: after an initial 10-min pre-incubation (activation of the Fast Start Taq DNA polymerase) and denaturation of DNA at 95°C, 45 amplification cycles were performed. Each cycle consisted of denaturation at 95°C for 10 s, annealing at 58°C for 10 s and extension at 72°C for 10 s. The fluorescence signals were measured after each annealing step.

By using the LightCycler-Relative Quantification Software provided by Roche, the amount of DNA encoding for *HER-2* genes is expressed as a relative ratio to a reference gene, which is normalized to a calibrator. The calculation of the *HER-2* DNA is based on the crossing point (Cp), which is determined by using the second derivative maximum method with the arithmetic baseline adjustment. After the determination of the Cp, the information is downloaded into LightCycler Relative Quantification Software, which automatically calculates the ratio between *HER-2* and the reference gene. According to the operator's manual, an experimental ratio between *HER-2* and the reference gene of less than 2.00 indicates that the sample is negative for HER-2 DNA over-amplification, while a ratio of greater than 2.00 indicates that the sample is positive for *HER-2* DNA over-amplification.

IHC

Immunohistochemical analysis was assessed with the polyclonal antibody A0485 (Dako Corporation, CA, USA). Staining was performed according to the protocol described in the manufacturer's guidelines as previously described [25]. Interpretation of IHC results was performed by two independent pathologists in two different Pathology Departments (L.K. and M.K.). Scores of 0 (no staining, or membrane staining in less than 10% of tumor cells) and 1+ (faint, interrupted membrane staining in more than 10% of the tumor cells) were considered negative for *HER-2* overproduction, scores of 2+ (weak to

moderate complete membrane staining in more than 10% of the tumor cells) were considered as indeterminate and scores 3+ (strong and complete membrane staining) were considered as positive.

CISH

CISH for *HER-2* was performed according to manufacturer's instructions (ZYMED, Montrouge, France), as previously described [12]. The CISH signals were evaluated using a NIKON Eclipse 400 microscope, equipped with a 40× dry objective lens. Cases with 1–5 gene copies per nucleus were defined as negative for amplifications; detection of 6–10 gene copies per nucleus in >50% of tumor cells was defined as low gene amplification (LGA) and 11–20 gene copies and/or large gene copy clusters per nucleus in >50% of tumor cells as high gene amplification (HGA) of *HER-2*. In order to confirm that the increased number of *HER-2* gene copies was due to amplification and not due to chromosome 17 polysomy, additional CISH analysis with a biotin labeled chromosome 17 centromeric probe was applied on adjacent sections in all cases with LGA. Chromosome 17 CISH analysis was performed according to ZYMED's protocol for formalin fixed paraffin embedded tissue sections.

Statistical analysis

Correlations between real-time PCR gene copy ratios, IHC and CISH were performed with the Kruskal–Wallis test, using Statmost statistical package (Statmost, DataMost Corp., USA).

Results

HER-2 status by real-time PCR on the LightCycler

The gene copy ratios obtained by real-time PCR for all samples ranged between 1.04 and 19.02. The calculated *HER-2*/reference gene ratios were significantly higher in IHC positive (3+) ($p<0.0001$) and CISH positive (HGA) ($p<0.0001$) cases than in the corresponding IHC negative and CISH negative cases. As can be seen in Table 1, results assessed as negative by CISH gave a median ratio of 1.67 (range 1.26–2.29) and results assessed as negative by IHC gave a median ratio of 1.7 (range 1.04–2.29). A ratio greater than 2.00 was observed in 19 (22.4%) out of 85 patients' samples.

Table 1
Real-time PCR *HER-2*/reference gene ratios in relation to IHC and CISH ($n=85$)

Group	*n* (%)	Real-time PCR			
		Median HER-2/reference gene ratios (range)	p [a]	Ratio <2.00 *n* (%)	Ratio ≥2.00 *n* (%)
IHC score					
0 or 1+	68 (80)	1.70 (1.04–2.29)	$p<0.0001$	64 (94.1)	4 (5.9)
2+	4 (4.7)	5.67 (1.99–8.02)		1 (25)	3 (75)
3+	13 (15.3)	2.64 (1.8–19.02)		1 (7.7)	12 (92.3)
CISH					
Negative	66 (77.7)	1.67 (1.26–2.29)	$p<0.0001$	62 (93.9)	4 (6.1)
LGA	10 (11.8)	2.63 (1.89–8.02)		2 (20)	8 (80)
HGA	9 (10.6)	2.64 (1.75–19.02)		2 (22.2)	7 (77.8)

LGA: low gene amplification.
HGA: high gene amplification.
[a] All comparisons used Kruskal–Wallis test.

Table 2
Comparison between IHC and CISH ($n=85$)

IHC score	CISH		
	Negative (*n*%)	Amplification (*n*%)	Total
(0, 1+)	65 (95.6)	3 (4.4)	68
(2+)	1 (25)	3 (75)	4
(3+)	0 (0)	13 (100)	13
total	66	19	85

Comparison of HER-2 gene amplification by real-time PCR and CISH

HER-2 gene amplification was measured in the same 85 samples by CISH. There was no amplification in 66 (77.7%) out of the 85 samples, while 19 (22.4%) out of the 85 samples showed *HER-2* gene amplification. In these positive samples, a low gene amplification (LGA) was observed in 10 (11.8%) out of the 85 samples and a high gene amplification (HGA) in 9 (10.6%) out of the 85 samples (Table 1).

Concordance rate between real-time PCR and CISH was 90.6%. Fifteen (79%) out of 19 CISH positive (LGA, HGA) samples had also a ratio greater than 2.00. Discrepancies were found among 4 samples, which were amplified by real-time PCR and did not amplify by CISH, as well as in 4 samples which were amplified by CISH but did not amplify, by real-time PCR (Table 1).

Comparison of HER-2 gene amplification by real-time PCR and HER-2 over-expression by IHC

All samples were also evaluated by IHC and scored for *HER-2* immunoreactivity. As shown in Table 1, 3 (75%) out of 4 and 12 (92.3%) out of 13 samples scored as 2+ and 3+ respectively, presented gene amplification (ratio ≥2.00) by real-time PCR.

Concordance rate between real-time PCR and IHC was 92.7%. There were discrepancies among 4 (5.9%) out of 68 samples that were scored as 1+ by IHC but showed *HER-2* gene amplification (ratio ≥2.00) by real-time PCR.

Comparison of IHC and CISH

Three (4.4%) out of 68 samples which were scored negative by IHC were found positive by CISH, while all 13 samples scored (3+) positive by IHC showed *HER-2* high gene amplification (HGA) by CISH (Table 2). Three (75%) out of 4 samples that were moderate by IHC (2+) showed LGA by CISH. Therefore, the concordance rate between IHC and CISH was 95.3%.

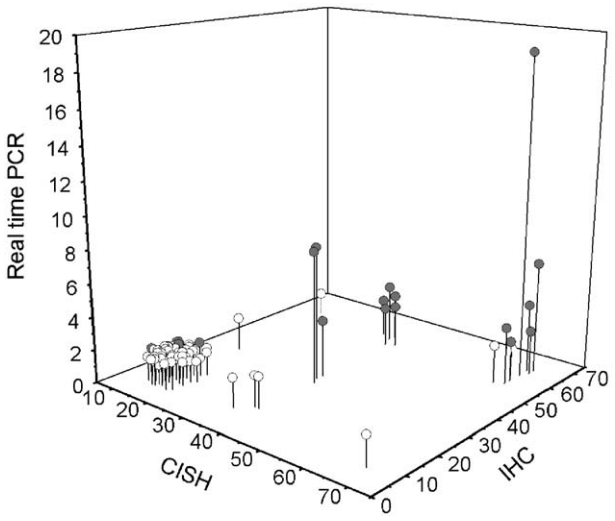

Fig. 1. Comparison of real-time PCR, CISH and IHC analysis of *HER-2* status in 85 breast cancer biopsies. Data are displayed as a function of real-time PCR, CISH and IHC values. Values with ratio ≥2.00 for real-time PCR (grey color) and ≥10 copies/nucleus for CISH were considered as positive. For IHC, values 3+ were considered as positive. Axis: *x* represents CISH (15–25: negative; 35–45: low gene amplification; 55–65: high gene amplification), *y* represents IHC (10–20: scores 0 or 1+; 30–40: scores 2+; 50–60: scores 3+) and *z* represents real-time PCR (ratios).

Correlation of HER-2 gene amplification by CISH, real-time PCR and HER-2 over-expression by IHC

There is a strong concordance between real-time PCR, CISH and IHC, since 15 samples were estimated as positive and 62 samples as negative by all three methods [(77/85, 90.6%)]. Sixty-eight samples were scored as 0 or 1+ by IHC; four (5.9%) out of these 68 samples showed *HER-2* gene amplification by real-time PCR (Table 1) and 3 (4.4%) of them were amplified by CISH (Table 2). Thirteen (15.3%) out of the 85 samples were scored as 3+ by IHC. All of them were amplified by CISH, while only 1 sample did not show *HER-2* gene amplification by real-time PCR. Moreover, there was only 1 sample among the 4 samples that were scored as 2+ by IHC, which did not show *HER-2* gene amplification by real-time PCR and CISH. On the other hand, there were 3 samples out of these 4 that were amplified by real-time and CISH (Tables 1 and 2). Results are illustrated graphically in Fig. 1.

Discussion

IHC is the most widely used method for *HER-2* status assessment since it has a relatively low direct laboratory cost, is relatively easy to perform and is easily adaptable by most Pathology Departments. However, the effects of tissue fixation and subjectivity of scoring remain troubling issues. FISH is considered to be the most accurate method and the gold standard for *HER-2* amplification. However, FISH is very expensive, more time consuming than IHC and requires special equipment. Recently, CISH that uses a robust and unique sequence probe developed for in situ hybridization has been introduced as a practical alternative to FISH in detecting *HER-2* amplification in paraffin-embedded breast carcinomas samples [8–14]. Despite the fact that CISH has a slightly lower sensitivity than FISH, it has numerous advantages since it is a relatively easy to use and low cost technique, and does not require expensive equipment as FISH. Real-time PCR has been compared recently in respect to IHC and FISH for determining *HER-2* status [22–24]. The high speed, automation and ease of real-time PCR make it feasible to test multiple samples for *HER-2* at the same time. Moreover, *HER-2* gene quantification by real-time PCR is highly sensitive, objective and offers good precision particularly when the robust mathematical model of efficiency adjusted relative quantification is used [24].

The present study reports for the first time the comparison of real-time PCR with CISH in order to assess *HER-2* status in 85 paraffin-embedded breast carcinomas from patients with early breast cancer. At the present, only differential PCR (dPCR) has been compared to CISH in order to verify the *HER-2* status [25]. The results demonstrate a very good concordance of real-time PCR with CISH (90.6%) and IHC (92.9%), which is in good agreement with previous reports (20, 23). In addition, we have found a high concordance between IHC and CISH (95.3%) and this is in keeping with the reported concordance values of 85%–95% when IHC is interpreted as either negative (0, 1+), or strongly positive (3+) [14,24].

When comparing real-time PCR, IHC and CISH, the concordance among them was 90.6% (Fig. 1). Real-time PCR could reveal 19 paraffin-embedded breast carcinomas from patients with early stage breast cancer in which the *HER-2* gene amplification was above the cut-off value (ratio ≥2.00). Seven (36.8%) of these cases strongly over-expressed *HER-2* protein (IHC score 3+) and showed high *HER-2* gene amplification with CISH. Interestingly, five among the remaining 12 breast carcinomas that were scored as 3+ by IHC, showed low *HER-2* gene amplification by CISH and three samples that were scored as 2+ by IHC showed low *HER-2* gene amplification by CISH. Surprisingly, there were four samples out of the 19 samples found positive with real-time PCR that did not amplify with CISH and did not over-express *HER-2* protein.

The mean ratios of these four samples (2.13±0.11, $n=4$) were very close to the cut off value of 2.00 (as proposed by Roche), while all the remaining positive samples had ratios >2.2 (range: 2.23–19.02, median: 3.81, $n=15$). Moreover, in a recent relevant study [24], Tse et al. considered a ratio >2.2 as being positive for HER2 gene amplification. It is also noteworthy that the relevant tissue sections were old and of poor quality, while FISH and CISH guidelines recommend that cut tissue sections should not be stored for more than 6–12 months.

The majority of the samples (15/19, 79%) that were classified as positive by real-time PCR were also positive with IHC and CISH. On the other hand, only one sample that was negative with real-time PCR was found positive with both IHC (3+) and CISH (HGA). There were four samples scored as 2+ by IHC; three of these samples were found positive and one did not show *HER-2* gene amplification with real-time PCR. In three samples, there was HER-2 gene amplification with CISH, while there was no amplification with real-time PCR. This

could possibly be due to a dilution of tumor cells with normal cells in the DNA preparation [20].

The assessment of HER2 status in breast cancer is critical for the management of advanced disease and therefore a priority for pathological standardization. This approach to therapy is heavily dependent on reliable and accurate laboratory results assessing the expression of HER2 in both the diagnostic evaluation and ultimately the selection of the most appropriate treatment for patients with breast cancer. Although FISH is currently regarded as the most accurate method and the gold standard for detecting HER-2 amplification and CISH has recently gained in popularity, we believe that real-time PCR is an alternative technique, given its many advantages.

In conclusion, our data obtained for amplification of *HER-2* status by real-time PCR on the LightCycler are comparable with those obtained by IHC and CISH. Our results confirm previous studies, which show that real-time PCR is more precise and reproducible than IHC. In comparison to CISH, we showed for the first time that real-time PCR gives comparable results. Since real-time PCR is reliable, semi-automated, and fast and moreover has advantages from an economical point of view, we recommend it for the determination of *HER-2* status.

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