Accurate breast cancer diagnosis through real-time PCR *her-2* gene quantification using immunohistochemically-identified biopsies

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Abstract. *her-2* gene amplification and its overexpression in breast cancer cells is directly associated with aggressive clinical behavior. The *her-2* gene and its Her-2 protein have been utilized for disease diagnosis and as a predictive marker for treatment response to the antibody herceptin. Fluorescent *in situ* hybridization (FISH) and immunohistochemistry (IHC) are the most common FDA-approved methodologies involving gene and protein quantification, respectively. False positive or negative *her-2*/Her-2 patient results may result in inappropriate treatment administration. To support accurate quantification and interpretation of results, in this study we have standardized qPCR analysis using previously identified IHC samples, obtaining very significant and clinically useful results.

Introduction

Breast cancer (BC) is one of the most common causes of mortality among females, accounting for 23% of total cancer cases and 14% of cancer mortality around the world (WHO, 2010). Lately, the number of BC fatalities has increased approximately to 40,000 annually in Mexico (http://www.sinais.salud.gob.mx/estadisticasportema.html). In human cancer, oncogenes such as *her-2* have been identified and used as molecular markers. *her-2* is one of the most frequently studied BC genes due to its overexpression observed in 20-30% of aggressive cases. High levels of *her-2* have also been found in several ovarian and endometrial tumors (1). Clinical studies have shown that *her-2* gene upregulation and Her-2 protein overproduction predicts poor prognosis in BC patients lacking estrogen and progesterone receptors, inducing a high rate of cell proliferation and tumor chemotherapy resistance (2,3). The discovery of *her-2* gene overexpression in BC tissues and its association with aggressive clinical behavior has generated diagnostic interest by using *her-2* gene expression as a predictive marker for herceptin treatment response (1-3). Immunohistochemistry (IHC) is the first methodology selected for Her-2 protein evaluation since it is the most economical; however, it is not the most precise. Females with an IHC score 3+ are candidates for the costly herceptin treatment. Samples with IHC score of 2+ are considered as inconclusive and a second evaluation with fluorescent *in situ* hybridization (FISH) methodology is required. FISH analysis is frequently omitted due to its high cost, the time-consuming methods involved and the shortage of specialized laboratories in which to perform it. The lack of a rapid, precise and economical *her-2*/Her-2 diagnostic test makes it necessary to implement alternative methodologies that have a lower cost, are less time-consuming and are more precise. In this study we standardize a quantitative polymerase chain reaction (qPCR) methodology that satisfies all premises mentioned before; being less expensive and less time-consuming than FISH and more precise than IHC.

Materials and methods

Tissue sample preparation and DNA biopsy extraction. Twenty-six biopsies of BC tissue fixed in formaldehyde and embedded in paraffin were previously analyzed by IHC. Eight of these samples were evaluated as IHC 3+, fifteen were evaluated as IHC 2+ and three of them were negative for Her-2 using the same methodology. All samples came from a private laboratory of clinical pathology in Ensenada (Baja California, Mexico). Biopsies were collected between 2004 and 2008 and stored at room temperature. Block samples were cut into 10-μm-thick sections and transferred into a sterile 1.7-ml tube. Paraffin was removed twice using 1 ml xylene for a 10 min incubation period and washed using 1 ml absolute ethanol, 1 ml 90% ethanol and 1 ml 70% ethanol, followed by 1 ml TE buffer. Tissues were pelleted following each step by centrifugation at 8,000 x g for 5 min. Final pellets were treated with 390 μl lysis

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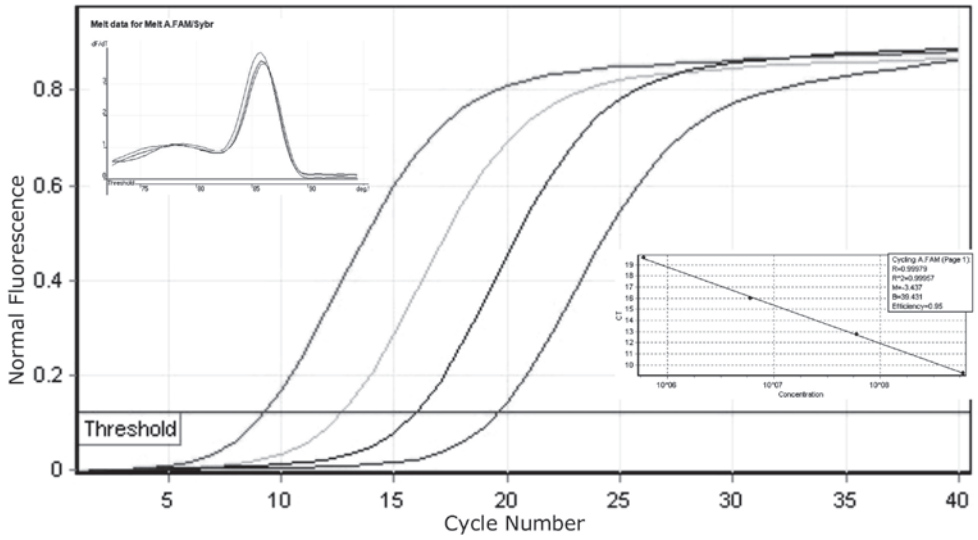

Figure 1. Plasmid dilutions presenting a strong correlation in *her-2* qPCR curve standardization. Correlation coefficient was >0.999 and the melting curves of *her-2* are shown as a single, sharp, narrow peak, indicating pure and homogeneous qPCR products were obtained. Similar results were obtained with *whn* gene control curve standardization (data not shown). qPCR, quantitative polymerase chain reaction.

buffer [150 mM NaCl, 15 mM EDTA, 60 mM Tris, (pH 8.0)], 50 μl 10% SDS, 25 μl lysozyme at 10 mg/ml, 10 μl RNase at 10 mg/ml and incubated at 37˚C for 30 min. Proteinase K digestion was carried out overnight out using 1 mg/ml of the enzyme at 37˚C (4). Chromosomal DNA was extracted by the standard protocol of phenol/chloroform/isoamyl alcohol and stored at -20˚C until use. Quantification of DNA molecules extracted from paraffin-embedded tissue samples was estimated by spectrophotometer at 260 nm (5).

The study was approved by the ethics committee of the Center for Scientific Research and Education (CICESE), Ensenada, Baja California, Mexico.

Control curve standardization. PCR products from *her-2* (103 bp) and winged helix nude (*whn;* 93 bp) control gene were obtained using fresh placental-extracted DNA and her-2-F, 5'-AAC TGG TGT ATG CAG ATT GC-3' and her-2-R, 5'-AGC AAG AGT CCC CAT CCT A-3'; whn-F, 5'-GGT GGA ATG ACC GAA GCT AT-3' and whn-R, 5'-GTC CTT CTG TGG CTC AAT CT-3'; primers previously published (6,7). PCR products were cloned using a pGEM vector (Promega®; Madison, WI, USA) and positive clones were grown in LB broth medium with 50 μg/ml ampicillin. Plasmid DNA was purified using Qiagen® miniprep kit (Hilden, Germany) and its concentration was measured by spectrophotometry assuming 1 OD at 260 nm = 50 μg/ml DNA (5). Serial dilutions from 10^{10}-10^5 molecules were prepared. Dilutions were used as plasmid copy number standards to generate a control curve and to quantify paraffin extracted *her-2* chromosomal DNA copies (Fig. 1). General formula used: (6.02 x 10^{23} copies/mol) x (concentration in g/μl) / (MW in g/mol) = copies/μl.

qPCR experimental conditions. Primers were obtained from Allele Biotech (San Diego, CA, USA). For amplification and data collection we used the Rotor-Gene 2000 real time cycler and software (Corbett Research; Sydney, Australia). Reactions were carried out in triplicate in a total volume of 25 μl. Each reaction mixture contained 1X PCR mix Rotor-Gene SYBR-Green, Qiagen®; 4 ng of each primer and 87 ng of template DNA final concentration. Cycling conditions were 95˚C for 5 min, 40 cycles; at 95˚C for 20 sec; at 60˚C for 20 sec and at 72˚C for 20 sec. Standard curves were prepared using 5 μl of each plasmid dilution and 20 ng of each primer.

qPCR her-2 gene quantification. *her-2* gene quantification in negative and positive (IHC 2+, IHC 3+) patient samples were obtained by calculating the ratio between *her-2*/*whn* gene amplification parameters. According to commercial protocols *her2*/*whn* negative control must present a value <2.0. Patient sample ratios with <2.0 values were also regarded as negative for *her-2* amplification. Patient sample ratios with >2.0 values were regarded as positive for *her-2* gene amplification.

Results and Discussion

Twenty-six BC biopsies previously evaluated by IHC were analyzed using a qPCR methodology. Biopsies 49, 50 and 51, reported as negative in IHC evaluation, also presented negative results in the qPCR analysis developed in this study (Table I). Fifteen biopsies were evaluated as IHC 2+ and eight were evaluated as IHC 3+ for Her-2 protein production (Table I). Biopsies 11 and 29 that were previously evaluated as IHC 3+ presented a <2.0 qPCR ratio in our evaluation. This result is considered as *her-2* negative when using commercial kits (e.g., *her-2*/*neu* DNA Quantification Assay; Roche Molecular Biochemicals, Mannheim, Germany). In this sense, samples 11 and 29 possibly demonstrated a polysomic condition because high qPCR values were obtained by *her-2* and *whn* genes individually (data not shown). In other words, the *whn* reference gene is located on the same chromosome as the *her-2* gene, therefore in a polysomic condition the *her-2*/*whn* ratio will always be around the value of 1.0 (Table I). It has been reported by FISH that *her-2* gene amplification occurs

Table I. qPCR results from formalin-fixed and paraffin-embedded breast biopsies tumors.

ID	Year	Patient age (years)	IHC	Lowest qPCR ratio	Highest qPCR ratio	Average ratio	SD
1	2004	72	3+	10.87	11.98	11.43	0.78
6	2004	47	3+	10.34	10.51	10.43	0.12
11	2004	44	3+	0.90	1.19	1.05	0.21
14	2005	55	3+	3.46	4.00	3.73	0.38
19	2005	59	3+	9.02	10.24	9.63	0.86
29	2005	75	3+	1.91	1.86	1.89	0.04
31	2005	35	3+	7.29	8.24	7.77	0.67
64	2008	54	3+	10.04	9.95	10.00	0.06
3	2004	58	2+	1.01	1.49	1.25	0.34
5	2004	53	2+	3.19	3.64	3.42	0.32
7	2004	46	2+	1.44	1.20	1.32	0.17
8	2004	51	2+	5.24	4.96	5.10	0.20
25	2005	47	2+	4.50	3.80	4.15	0.49
35	2005	45	2+	7.11	4.37	5.74	1.94
65	2008	50	2+	1.54	1.70	1.62	0.11
68	2009	35	2+	3.96	3.64	3.80	0.23
70	2009	57	2+	3.33	2.70	3.02	0.45
71	2007	90	2+	2.42	3.41	2.92	0.70
73	2008	38	2+	3.82	2.78	3.30	0.74
74	2008	-	2+	9.80	9.85	9.83	0.04
75	2008	50	2+	3.35	3.49	3.42	0.10
77	2008	73	2+	1.59	1.58	1.59	0.01
78	2008	52	2+	50.40	52.49	51.45	1.48
49	2006	55	neg	1.14	1.42	1.28	0.20
50	2006	56	neg	0.50	0.61	0.56	0.08
51	2006	80	neg	1.00	0.62	0.81	0.27
Control				0.92	1.08	1.00	0.11

IHC, immunohistochemistry; qPCR, quantitative polymerase chain reaction; SD, standard deviation.

on its own chromosome or have chromosome 17 polysomy, the latter being always reported as negative by qPCR (8). In addition, FISH analysis depends on the number of *her-2* positive cells observed with respect to the amount of tissue analyzed. In this sense, qPCR analysis avoids this possible misinterpretation during ratio calculation among *her-2*/*whn* genes as independent from tissue amounts.

In the IHC 3+ samples, sample 14 presented a 3.73 average qPCR ratio. Most of IHC 2+ biopsies evaluated in this study and reported in Table I, presented a similar 3.5 average qPCR ratio, meaning that biopsy 14 should be considered as an IHC 2+ and not an IHC 3+ sample. In addition, IHC 3+ samples 1, 6, 19, 31 and 64 presented a 9.5 qPCR average ratio, making these results most reasonable for an IHC 3+ evaluation performed by a pathology laboratory (Table I).

Previous studies have shown an equivalence of 80-90% among FISH and qPCR techniques on samples assessed as 3+ by IHC (9,10). In this sense, our results confirm the same percentage of accuracy, as we only had one discrepancy among the eight samples analyzed (ID 14). The discrepancies in samples 11 and 29 were caused by polysomy of the *her-2* gene on chromosome 17, not IHC or qPCR misevaluation. However, special attention must be given to polysomy when qPCR is the only methodology utilized. Generally, high qPCR values will be found for *her-2* and *whn* genes individually, contrary to low qPCR values obtained in negative or IHC 2+ samples.

Conversely, great differences have been reported between IHC 2+ results with respect to FISH and qPCR evaluations (9-11). It has been mentioned that IHC 2+ result variability is principally caused by tissue quality, fixation time, type of antibody used and lack of standardization among users (3,12). In this sense, our experience indicates that IHC 2+ misevaluation depends on low Her-2 protein levels contained in samples analyzed, but principally on resolution capacity limitations of IHC methodology to quantify low target protein levels. It is important to consider that IHC 3+ sample biopsies were also evaluated by the same pathology laboratory and few discrepancies were obtained.

Discrepancies of 20-40% from samples evaluated as IHC 2+ with respect to results obtained by using FISH or qPCR techniques have been reported (11,13-17). In this study, from fifteen biopsies evaluated as IHC 2+, four (ID 3, 7, 65

and 77) were identified with a <2.0 qPCR ratio, showing non-amplification differences with respect to the negative control (Table I). In addition, some researchers have suggested that qPCR cut-off should be greater than 2.7 to be considered as *her-2* amplification (11,16,18). In this sense, we suggest that qPCR ratios between 2.5 and 5.0 must be considered as an IHC 2+ result. In this study, almost 50% of IHC 2+ biopsies analyzed presented a 3.5 average qPCR ratio that adequately matched our proposal. However, samples 8, 35, 74 and 78, also reported as IHC 2+, presented a >5 qPCR ratio, meaning that these samples must be classified as IHC 3+ instead of IHC 2+. In this case, a misevaluation occurred when using IHC methodology with respect to qPCR, even when these biopsies presented high Her-2 protein levels. Taking into account samples 8, 35, 74 and 78, presenting a >5 qPCR ratio, and samples 3, 7, 65 and 77, presenting a <2 qPCR ratio, more than 50% of biopsies identified as IHC 2+ were wrongly classified, displaying a higher percentage discrepancy with respect to data reported (3,11,13,16). The results obtained in this study suggest a new IHC-qPCR relationship, which could be used to easily differentiate among IHC negative, IHC 2+ and IHC 3+ results. IHC negative result samples should demonstrate a qPCR ratio <2, IHC 2+ result samples should demonstrate ratios between 2.5 and 5.0, and IHC 3+ result samples should demonstrate a qPCR ratio >5. This proposal is in concordance with biopsies and qPCR results obtained in this manuscript.

In this study, qPCR technique has been shown to be complementary to IHC analysis, similar to FISH; however, qPCR analysis is faster, less expensive and is an equally useful alternative methodology for patients.

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