RESEARCH ARTICLE

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Prospective Validation of a One-Step RT-qPCR-based Test for Quantifying *HER2* Gene Expression in Breast Cancer

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Abstract

Objective: To Validate a one-step RT-qPCR as a reliable diagnostic tool in *HER-2* positive breast cancer. Further, establishing this tool as a standard procedure to quantify HER-2 expression in Breast cancer patient. **Methods:** Here we report a prospective validation study that shows the concordance of one-step RT-qPCR in assessing the *HER2* levels in formalin-fixed paraffin-embedded (FFPE) tissue samples with the current paradigm of diagnosis such as Immunohistochemistry (IHC) and Fluorescence-in-situ Hybridization (FISH). We collected 275 FFPE samples from the Department of Pathology, Ibn Rochd University Hospital Center. IHC was carried out in all the samples and those with a score of 2+ were also analyzed using FISH. We extracted mRNA from FFPE samples and performed RT-qPCR, and the results were compared with those obtained using IHC/FISH. *HER2* mRNA levels were quantified and normalized using the reference genes *RPL30* and *RPL37*, based on our earlier reports. **Results:** HER2 cut-off value was fixed at 11.954 corresponding to the combination of the best sensitivity and specificity (93.4% and 100%) respectively, and with positive predictive values PPV that reached 100% and a negative predictive value NPV that reached 89.4%. The results showed 100% concordance with FISH. The Kappa coefficient was 0.863 which indicated concordance with IHC. The area under the curve (AUC) which is an important parameter that determines the diagnostic accuracy of the test was calculated as AUC=0.955. The results were comparable to some of the recent studies published in a similar direction. **Conclusion:** Our study shows that one-step RT-qPCR-based quantitation is an accurate and reproducible test to record *HER2* gene expression for a better treatment orientation focusing on maximal therapeutic and survival outcome.

Keywords: Breast cancer- HER2- one-step RT-qPCR- immunohistochemistry- fluorescence in situ hybridization

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Introduction

Despite the theragnostic advances made so far, breast cancer remains the second most common cause of mortality among women after lung cancer and the most common cancer affecting women worldwide [1]. It is a highly heterogeneous cancer in terms of histology, presentation, molecular profile, disease progression, etc., which makes the treatment options and clinical outcomes different for each subtype [2]. The National Cancer Institute (NCI) estimates that more than 297,000 women in the United States will be diagnosed positive for breast cancer by the end of the year 2023, and more than 43,000 deaths will be registered [3]. Most importantly, although recent reports indicate that the rate of breast cancer in developing countries is less compared to that of developed ones, there has been an alarming increase in the mortality rate in low- and middle-income countries (LMIC), which may be largely attributed to the lack of accurate screening and diagnostic procedures [4].

Invasive breast cancer is categorized into five different molecular subtypes based on the expression of certain genes in the cancer cells, which determines their clinical behavior [5]: (i) Luminal A breast cancer cells express estrogen (ER) and progesterone receptors (PR). Since they have low levels of *Ki-67*, and do not express Human epidermal growth factor 2 receptor (HER2), the cells tend to grow at a slower rate compared to other subtypes and have a good prognosis, (ii) Luminal B breast cancer is positive for ER and negative for *HER2, PR* and the cells have high expression of *Ki-67*, (iii) Luminal B-like breast cancer cells express both *ER* and *HER2* and may be either

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PR positive or negative. The cells also have different *Ki-67* cells (either high or low), and hence this type of cancer has a slightly worse prognosis compared to luminal A cancers, (iv) *HER2*-enriched subtype is *ER* and *PR* negative. Although the prognosis is worse compared to the luminal A subtype, timely treatment with *HER2*-targeted therapies has shown successful treatment outcomes, (v) Triple-negative or basal-like subtype breast cancer cells do not express *ER, PR*, or *HER2*. The incidence of this subtype is mostly reported among young women who have a BRCA1 mutation, and it is more aggressive compared to luminal subtypes [6].

Approximately 20 to 30% of breast cancer cases have *HER2* gene amplification and hence HER2 receptor overexpression [7]. *HER2* (185kD) is a 1255 amino acid long transmembrane tyrosine kinase that belongs to the family of epidermal growth factor (EGF) encoded by the *ERBB2* gene (also named *HER2, neu*, or *p185*) and was discovered in the year 1984. Being a tyrosine kinase, HER2 overexpression is implicated in rapid cell division which drives the oncogenesis process. *HER2* gene is located on the long arm of chromosome 17 (17q12). The clinical diagnosis of HER2-positive breast cancer is most often performed by immunohistochemistry (IHC) or fluorescence in situ hybridization (FISH). The discovery of trastuzumab (Herceptin) almost 25 years ago revolutionized the treatment of *HER2*-positive breast cancer [8]. It is the first-line treatment option and is also used as an adjuvant for combined therapy with either paclitaxel, docetaxel, or carboplatin [9].

American Cancer Society recommends that all invasive breast cancers should be tested for the presence of *HER2*. Currently FISH remains the gold standard for *HER2* diagnosis, whereas IHC is more popular in preclinical settings since it is easy and cost-effective compared to that of FISH [10]. However, a definitive diagnosis is not currently possible by IHC. Although FISH is a highly sensitive and accurate method for diagnosing *HER2* amplification, the prohibitive cost, and the requirement of specialized laboratory facilities, tools, and expertise makes it less attractive for low-resource settings [11]. Moreover, using FISH, the optimization of tumor morphology is difficult, and it is also challenging to differentiate between the diverse types of carcinomas. The rapid quenching of the fluorescent probes at room temperature is also a potential disadvantage of FISH. Furthermore, using the current techniques, it is not possible to stratify patients with lower ranges of *HER2* expression [12]. The false negative results may lead to trastuzumab-induced cardiotoxicity and unnecessary financial burden, whereas false negative results may cause loss of quality-of-life years (QALY), metastasis, disease recurrence, and burden of additional treatment costs [13]. It is also impossible to evaluate the tumor progression from the results of diagnosis using either FISH or IHC, which is a crucial factor before the administration of the treatment [14]. There is also a discrepancy reported in the *HER2* expression in the tumor and the affected lymph node [15]. These limitations urge the need for more valid, quantitative, and reliable standardized assays for the definitive diagnosis of HER2-positive breast cancer. Since *HER2* gene amplification is correlated with the over-expression of mRNA and protein levels, it is possible to measure the *HER2* at DNA, mRNA, and protein levels. Quantitative real-time reverse transcription-PCR (RT-qPCR) is an advanced PCR technology that allows reliable and rapid detection and quantification of gene products at each PCR cycle, which enables the quantification of *HER2* gene products [16]. Apart from absolute and relative quantification of gene expression it is also widely used for identifying circulating tumor cells, validating DNA microarray results, single nucleotide polymorphism (SNP) discovery and validation, assessing viral, bacterial, and fungal loads, etc [17–20]. Previous analysis performed in a retrospective study has shown that RT-qPCR could be used as a possible technique for accurate quantification of *HER2* using breast cancer biopsy samples [21]. The results indicate that RT-qPCR-based tests could bring a paradigm shift in the current diagnosis workflow of *HER2*-positive breast cancer. Moreover, in contrast to the current dosing regimen which is primarily based on the weight of the patient, the new dosing regimen advocated by several research groups worldwide based on pharmacokinetics, quantitative *HER2* expression, etc. necessitates new strategies for *HER2* quantification such as RT-qPCR in the clinics [22]. It will also channel the intelligent use of the drug to reduce both the financial and clinical toxicities associated with the current use of trastuzumab.

Materials and Methods

Samples

Altogether 275 tumor samples were collected from breast cancer patients who reported/diagnosed at the Ibn Rochd University Hospital Center (Casablanca, Morocco), after their informed consent. The tumor samples obtained from the Pathology Department of the Ibn Rochd University Hospital Center were formalin-fixed and paraffin-embedded (FFPE) and collected prospectively from the patients over a period of one year. The sample collection and the study outlined in this article were approved by The Institutional Ethics Committee.

Cell lines and cell culture

Breast cancer cell lines used in this study (*HER2* over-expressing SKBR-3, ATCC® HTB-30™, and *HER2* low MCF7, ATCC® HTB-22™) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SKBR3 cells were cultured in ATCC-formulated Modified McCoy's 5a medium and MCF7 cells in ATCC-formulated Eagle's Minimum Essential Medium respectively. The media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/ streptomycin antibiotic solution as directed by ATCC, and the cells were cultured at standard culture conditions (37°C, 5% CO_2) and sub-cultured when the cells reached ~80% confluence.

Immunohistochemistry (IHC)

IHC was done at the Pathology Department of the Ibn Rochd University Hospital Center. VENTANA anti-*HER2*/neu (4B5) Rabbit Monoclonal Primary

Antibody was used for the IHC studies on a fully automated BenchMark IHC automat. The results were reported in accordance with the 2018 ASCO/CAP recommendations [23].

Fluorescence in situ hybridization – FISH

Tissue samples which were scored 2+ were validated using FISH. 3 µm paraffin tissue sections were used for FISH analysis. HER2 FISH pharmDx™ (Dako Denmark A/S, Glostrup, Denmark) probe was used to perform the FISH, and the images were captured using a fluorescence microscope with a suitable filter. HER2 amplification was reported as per 2018 ASCO/CAP recommendations [23].

RNA extraction from cell lines

RNeasy Mini kit from QIAGEN was used for extracting RNA from SKBR3 (HER2high) and MCF-7 cells (HER2low) as per instructions from the manufacturer. Approximately 30µl of RNA was eluted in RNase free water and the samples were stored at -80°C, until used. The quality and concentration of RNA were recorded in a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

Deparaffinization of FFPE tissue and total RNA extraction

PureLink™ FFPE RNA Isolation Kit (Invitrogen, Carlsbad, California) was used to remove paraffin and extract total RNA from tissue samples. Two to three 10-µm tissue sections were used for RNA extraction according to the instructions from the manufacturer. Approximately 30-50µl RNA was eluted in RNase free water (according to the quantity of the tissues) and stored at −80°C until used. The quality and concentration of RNA were recorded in a NanoDrop ND-2000 spectrophotometer as mentioned earlier.

Reverse transcription-quantitative realtime- PCR
Primers and probes design

Primer 3 plus software version 2.0 was used to design the primers and probes sequences. The probes were manufactured by Eurofins (Germany) with a 5′6- carboxyfluorescein (FAM) for *HER2* and the two control genes *RPL30 (FAM)* and *RPL37 (VIC)* as reporter dyes, and a 3′Black Hole Quencher-1 (BHQ-1) as a quencher dye for both the probes. The primers were designed to exclude the amplification of genomic DNA. The specificity of the primers and probes was validated using the *HER2* mRNA levels in MCF-7 (HER2low) and SKBR3 (HER2high).

One-step RT-qPCR

HER2 mRNA levels were quantified using "one step" RT-qPCR in a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) following the relative quantification method, and the data analysis was done using QuantStudio 6 Flex software V1.0. The results obtained from one-step RT-qPCR were expressed as relative levels of HER2 mRNA with respect to the calibrator sample obtained from the *HER2*low MCF-7 cell line and normalized as *1X* expression of *HER2*. *HER2*high SKBR- 3 cell line was used as a positive control. TaqMan one-step Master Mix (Applied Biosystems) was used to perform one-step RT-qPCR as per the manufacturer's instructions. The Master Mix contained Amplitaq Gold hot start fast DNA polymerase and M-MLV reverse transcriptase. 10 µL RT-qPCR reaction mix contained Taqman 1-step master mix (4X, 2.5µL volume), primers (300 nM each), and probe (200 nM). A total of 6.5 µL of the total purified RNA (200ng/µL) was added to the 3.5µL PCR Mix. RT-qPCR reactions were performed in duplicates. The cycle conditions were maintained the same for the 3 transcripts at 50°C for 5min (reverse transcription reaction), 95°C for 20s (initial denaturation), and 50 cycles at 95°C for 15s. The annealing and extension steps were combined and performed at 60°C for a duration of 60s. For the relative quantification of *HER2*, the reference genes reported in our previous study were used, and the same protocol was used for quantifying *HER2* relative to the geometric means of the reference genes with respect to the calibrator sample and the errors were calculated as per the rules of error propagation [24]. The risk of contamination was eliminated by performing the experiments in a pre-PCR, a PCR, and a post-PCR room, wherein the sample transportation was performed via airlock windows. To rule out contamination, a negative control containing RT-qPCR reaction mix added to pure water was also kept.

Relative quantification = $2^{-\Delta\Delta Ct}$

$2^{-\Delta\Delta Ct}$ method was used for the relative quantification of *HER2* gene expression. The algorithm considered the calibrator gene expression (in this case HERlow MCF7) represented as 1X expression of *HER2*. *HER2* gene expression was calculated as per the following equation: fold induction = 2− [ΔΔCt], where ΔΔCt = [Ct *HER2* (Tumor sample) − Ct endogenous control (tumor sample)] − [CtHER2 (MCF7) − Ct endogenous control (MCF7)]. The ROC curve method was used for differentiating between *HER2* positive and negative samples. The RT-qPCR data obtained in this study were classified into two expression levels. Overexpression of *HER2* was categorized as positive cases, whereas normal expression of *HER2* was categorized as negative cases. A normalized ratio was used to represent the result and if the value was above the predetermined threshold, *HER2* was considered overexpressed in the samples.

Results

HER2 expression in breast cancer patients

The expression of *HER2* was assessed in all breast cancer patients using IHC, FISH, and qRT-PCR. The studies were performed in the Department of Pathological Anatomy, CHU Ibn Rochd, Casablanca. The baseline characteristics of the patients (275 patients) included in this study have been shown in Table 1. IHC was performed using paraffin-embedded tissue sections, and the representative image is shown in Figure 1 panel 1. *HER2* overexpression can be clearly visualized in amplified *HER2* infiltrating breast carcinoma (Figure 1a) as against minimal *HER2* expression in a non-amplified *HER2* infiltrating breast carcinoma (Figure 1b). *HER2*

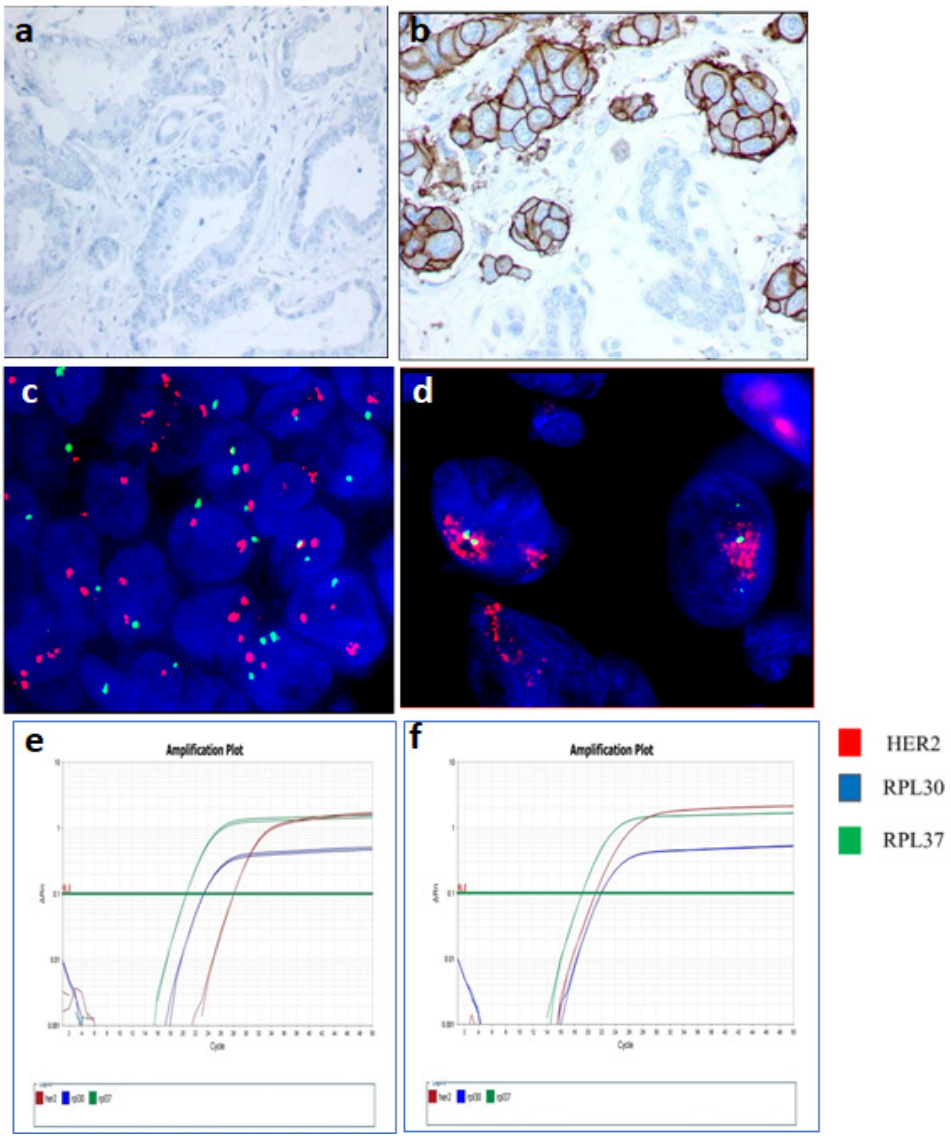
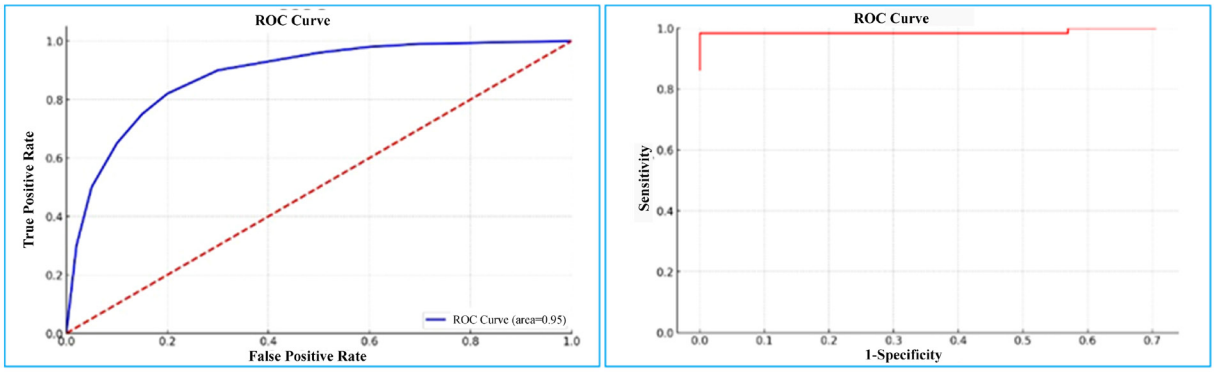


Figure 1. Examples of *HER2* protein Overexpression (IHC), *HER2* gene amplification (FISH) and mRNA quantification by RT-qPCR. Panel 1. Immunohistochemistry a. Sample showing no *HER2* overexpression in non-amplified *HER2* infiltrating breast carcinoma. b. Sample showing strong *HER2* overexpression in an amplified *HER2* infiltrating breast carcinoma. Panel 2. *HER2* gene amplification detected by FISH (red signals: HER2, green signals: centromeres of chromosome 17) c. Sample showing low level of *HER2* gene amplification, without clusters and two green signals from centromere of chromosome 17. d. Sample showing high level of HER2 amplification in HER2-infiltrating breast carcinoma which shows clusters of red signals, indicating *HER2* amplification in presence of polysomy of centromere of chromosome 17 (green signals). Panel 3. Amplification curves using RT-qPCR of *HER2* mRNA in two different samples. e. negative sample with normal *HER2* expression. d. positive sample with *HER2* overexpression.

gene amplification was also confirmed using FISH (Figure 1, panel 2), wherein the red signals indicate *HER2* amplification and green signals indicate centromeres of chromosomes. Figure 1c shows a sample with low/basal expression of *HER2* as indicated by the scattered red clusters and the presence of two green signals from the centromere of chromosome 17. In sharp contrast, a high level of *HER2*-infiltrating breast carcinoma sample (Figure 1d) showed *HER2* amplification as indicated by the clustering of red signals and the polysomy of centromere of chromosome 17 (green signals). Later, *HER2* mRNA expression was performed using "one-step" qRT-PCR after extracting the total RNA from all 275 FFPE tissues. The standard curve was plotted for *HER2* and the reference genes *RPL30* and *RPL37*, and the R2 value and slope indicated the efficacy of the RT-qPCR reaction, and also the primers and probes of the target genes. Figure 1 Panel 3 shows the amplification curves of *HER2* mRNA in two different samples using RT-qPCR, wherein Figure 1e is the negative control which had normal expression of *HER-2*, and Figure 1d is the sample with over-expression of *HER2*. The assay reproducibility was determined on 3 pools of *HER2*high patient samples (3 levels of mRNA overexpression: high, medium, and low) and one pool of *HER2*low patient samples with a 10-fold passage number. Repeatability was within the set analytical objectives (CV<5%).

Sensitivity and specificity of the test by ROC analysis

The ROC curve analysis which is the receiver



CUT OFF	VP	FP	FN	VN	TOTAL	SPEC	1-SPEC	SENS	VPP	VPN
0.008	IN	38	65	0	1	104	0.015152	1.000000	0.368932	1.000000
0.500	IO	37	39	1	27	104	0.409091	0.973684	0.486842	0.964286
11.954	IY	34	0	4	66	100	1.000000	0.934737	1.000000	0.942857

Figure 2. Sensitivity and Specificity of the Test by ROC Analysis: The ROC curve is used to determine both sensitivity and specificity of the test. The optimal cut-off value in our context is the value of 11.954 corresponding to the combination of the best sensitivity and specificity (93.4% and 100%). The area under curve which is a parameter to determine the diagnostic value of the test was also calculated (AUC=0.955).

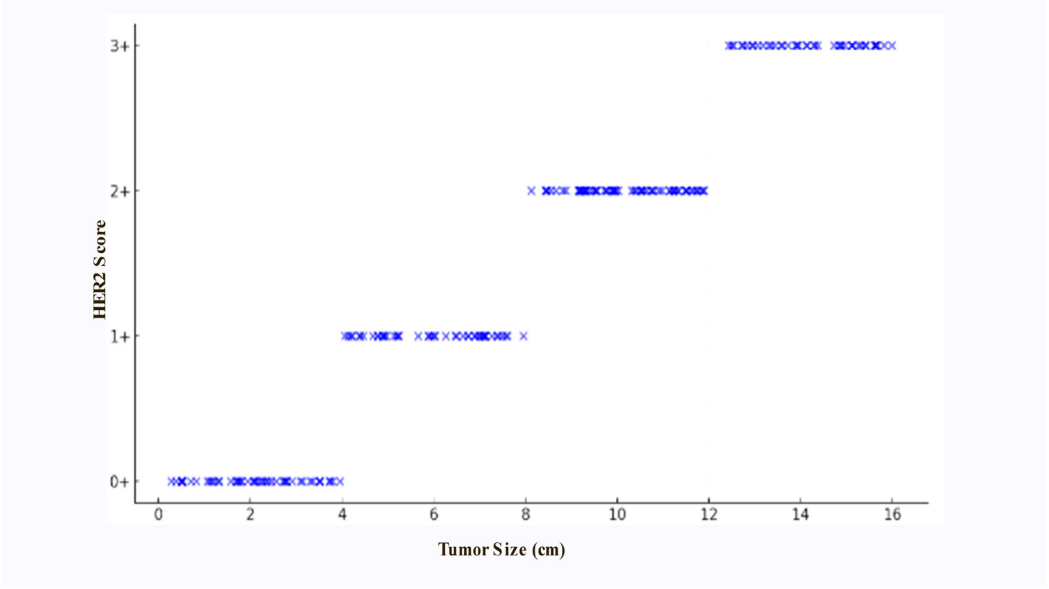


Figure 3. Relationship between Tumor Size and *HER2* Score. The graph illustrates the relationship between tumor size and HER2 score in 269 patients.

operating characteristic curve indicates the true positive cases against false positive cases. ROC curve was plotted for the samples using the *HER2* mRNA levels obtained using RT-qPCR and IHC score to determine the optimal diagnostic cut-off value with minimized false-negative and false-positive (Figure 2). The area under the curve (AUC) which is an important parameter that determines the diagnostic value of the test was calculated as AUC=0.955. A test with a strong discriminative power must have an AUC value close to 1 and far from 0.5, and our results were close to 1, which indicates the diagnostic accuracy of RT-qPCR. As per the ROC analysis, the optimal cut-off value in our context was 11.954 corresponding to the combination of the best sensitivity and specificity (93.4% and 100%) respectively, and with positive predictive values PPV that reached 100% and a negative predictive value NPV that reached 89.4%.

The concordance between IHC vs RT-qPCR and FISH vs RT-qPCR is shown in Table 2. The results show that among the 211 samples that were IHC negative, RT-qPCR was negative for 209 samples and positive for 2 samples. Similarly, among the 53 samples that were positive as per IHC, RT-qPCR was negative for 9 samples and was positive only for 44 samples. The Cohen's Kappa coefficient was around 0.863 which indicates a very good concordance between IHC and RT-qPCR tests. FISH vs RT-qPCR results showed 100% concordance since 11 samples that were negative in FISH were also RT-qPCR negative. Hence the results indicate that IHC and RT-qPCR were consistent in assessing the molecular status of the patients in the study. High concordance also indicates that depending on the availability and feasibility,

Table 1. Baseline Characteristics of Patients Included in This Study

Characteristics		Number of patients	Percentage
Age	20-30	4	1.45
	31-40	33	13.82
	41-50	107	38.91
	51-60	65	23.64
	>60	61	22.18
SBR Grade	Grade 1	17	6.18
	Grade 2	152	55.27
	Grade 3	106	38.55
Presence of	Oui	130	47.27
emboli	Non	145	52.73
Tumor size	<1cm	36	13.09
	1-5cm	221	80.36
	5-10cm	2	0.73
	>10cm	16	5.82
RE%	Negative (<1%)	62	22.55
	Positive (>1%)	213	77.45
RE intensity	Intense	123	44.73
	Moderate	67	24.36
	Negative	85	30.91
RP%	Negative (<1%)	75	27.27
	Positive (>1%)	200	72.73
PR intensity	Intense	95	34.55
	Moderate	72	26.18
	Negative	108	39.27
HER2 SCORE	0+	133	48.36
	1+	62	22.55
	2+	25	9.09
	3+	55	20.00
KI-67%	High (>10%)	241	87.64
	Low (<10%)	34	12.36
T,n	pT1	174	63.27
	pT2	99	34.91
	pT3	2	0.73
N, n	pN0	154	56
	pN1	78	28.36
	pN2	31	11.27
	pN3	12	4.26
M, n	0	275	100
pStage	Stade I	117	42.55
	Stade II	114	41.46
	Stade III	43	15.63
	Stade IV	1	0.36

one technique may be used instead of the other, and the treatment decisions will generally be similar based on the results, although the patient's overall clinical context is also a crucial factor in determining the treatment.

The HER2 expression studies showed that larger tumors were associated with higher *HER2* scores (Figure 3), most likely due to the higher expression of *HER2*, which in turn might have fueled an aggressive tumor growth. However, we did observe a certain degree of variability in this study, since some of the medium-sized tumors showed higher HER2 scores, whereas certain other tumors with approximately same size showed low *HER2* scores.

Table 2. Concordance between IHC vs RT-qPCR / and FISH vs RT-qPCR in Our Study

	RT-qPCR Negative	RT-qPCR Positive
IHC negative	209	2
IHC positive	9	44
Kappa coefficient	0.863	
FISH negative	11	11
FISH positive	0	0

Figure 4 shows the relationship between *HER2* score and SBR grade. *HER2* score and SBR grade are two key parameters for characterizing breast tumors. The results indicate that there is an association between *HER2* score and SBR grade, two key diagnostic criteria in breast cancer. The observation of an association between these two parameters, although not perfectly linear, could suggest that patients with *HER2* overexpression also tend to have histologically more aggressive tumors.

Discussion

HER2 amplification status is a crucial factor in the response to targeted anti-*HER2* therapy, and hence there is a pressing need to streamline the therapy based on the differential expression of *HER2* and the underlying signaling mechanisms. There is ample evidence that indicates *HER2* levels can predict the response to anti-*HER2* therapy, and survival outcomes [25]. Although FISH is a gold standard technique to assess the amplification of *HER2*, *IHC* is more frequently used, the latter being a comparatively inexpensive technique [10]. However, IHC has a high interoperator variability and a high rate of false positives and false negatives [26]. Despite the specificty imparted by the antibodies, the assay positivity in IHC is largely dependent on the enzymatic activity of Horseradish peroxidase (HRP) which is conjugated to the antibodies. The staining intensity is significantly influenced by the quality and concentration of HRP and substrate, and also storage conditions, and duration of incubation [27].

The main focus of this study was to evaluate the feasibility of one-step RT-qPCR to be used as a test for quantifying the *HER2* gene expression in breast cancer and validate the results with those obtained using FISH and IHC. *HER2* overexpression in the breast cancer biopsy samples was confirmed using IHC, and classified as negative (score 0/1+), or positive (score 3+), as per the standard guidelines [23, 28]. If the score obtained was 2+ another method of reference i.e FISH was also used. However, FISH has its own limitations, apart from being an expensive technique, the fluorescence quenching happens fast, and hence the slides cannot be preserved for long [29]. Moreover, the facilities to carry out FISH

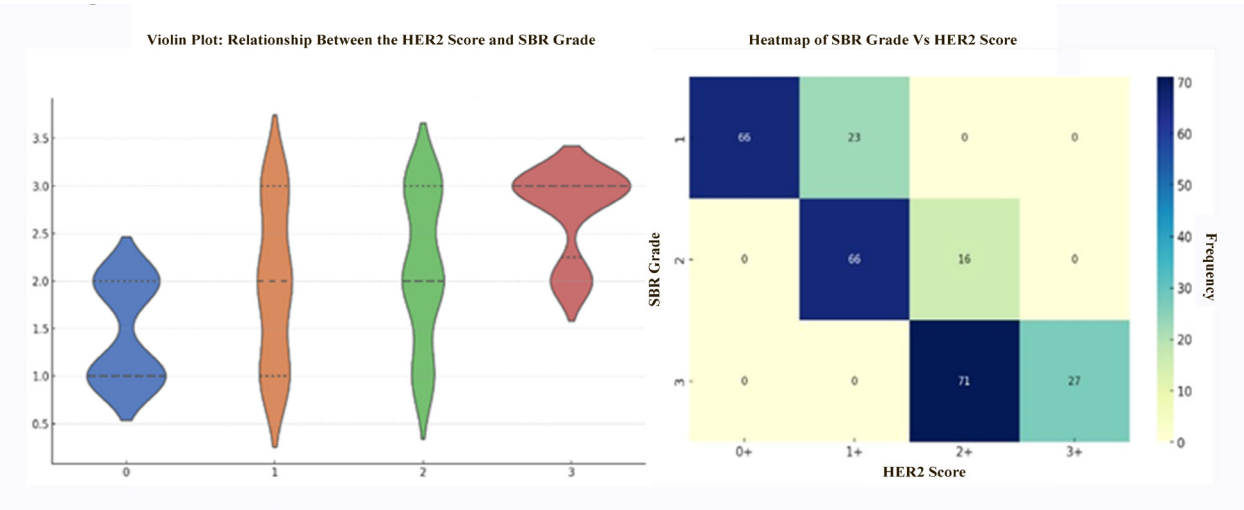


Figure 4. Relationship between *HER2* Score and SBR Grade

may not be available at all laboratories. This necessitates a sensitive, quantitative, rapid, and accurate test to standardize and automate the diagnosis of *HER2+ve* breast cancer.

Previous studies on the use of RT-qPCR for quantifying *HER2* were contradictory, and even certain studies do not recommend its use [30, 31]. We presume that this is largely due to the mRNA degradation that might have occurred while its extraction from formalin fixed paraffin embedded (FFPE) tissues, or the absence of suitable reference genes. However, in the current study, we could overcome the discrepancies in the results by using appropriate endogenous controls/reference genes, thereby making the results more accurate. We used *RPL30* and *RPL37* as endogenous controls which we selected based on our previous results [24]. We carried out clinical validation of one-step RT-qPCR using FFPE samples and compared the results with those of IHC and FISH. The first step was to identify an optimal diagnostic cut-off value using ROC-based analysis and using the IHC reference score. We found that an optimal value of 11.954 represented the best specificity (100%) and sensitivity (89.4%), and an AUC value of 0.955. The optimal cut-off value thus indicates that if the *HER2* expression is above 11.954, it is considered positive and if the expression is below 11.954, the sample is HER2 negative. Our study showed a high concordance of RT-qPCR and IHC results. Cohen's Kappa coefficient of 0.863 (Table 2) indicates very good concordance between IHC and RT-qPCR tests, which in turn suggests that the two methods are largely consistent in their assessment of the clinical status of patients in the study. Furtermore, a 100% concordance obtained with FISH further validates the use of RT-qPCR. A high concordance also reinforces the reliability of both diagnostic methods in the clinical context, which indicates that the healthcare professionals can rely on either of these tests to provide accurate information about patients' disease state, depending on the availability and/or feasibility. However, we did observe a discordance for a few cases (11 patients in our study), which necessitates re-evaluation of the results and consideration of other pathophysiological factors. So far there are no elaborate studies which show a similar trend, and we believe that further studies in this direction may have profound clinical implications especially in personalizing the treatment approach.

Our results are in conjunction with several other studies published in this direction which show concordance of RT-qPCR with IHC/FISH. In a very recent study conducted by Li et al. using 323 patient samples which included different molecular subtypes, a high concordance (89.4%) was found to exist between IHC and RT-qPCR in the case of *HER2* expression [32]. Previously, Chen et al. conducted studies using 397 breast cancer patient samples of different subtypes and found that for *HER2*-positive cases there was 81.6% concordance between RT-qPCR and IHC. Their study recommends the use of RT-qPCR as a complementary method to molecular subtyping under circumstances of ambiguous IHC results [33]. Caselli et al. have conducted studies using different subtypes of breast cancer and showed that the mRNA level expression of breast cancer biomarkers such as *HER2, ER, PR*, and *Ki67* highly correlated with that of IHC. Most importantly, RT-qPCR results were reproducible, and offered a higher degree of standardization for *Ki67*, and also solved *HER2* cases that were uncertain as per IHC/FISH assessment [34]. Similarly, a high degree of concordance i.e., 94% was observed by Gheni and Westenberg in their studies using 54 paired tissue samples (FFPE tissues). Their study concluded that IHC can be used only for initial screening and a more accurate, quantitative and a reproducible result may be obtained by using RT-qPCR in routine clinical practice [35]. Al Banyahyati also reported that one-step RT-qPCR method was quite accurate in giving correlated results with those of FISH/IHC methods, and even recommended using the former as the initial choice of diagnosis for *HER2* amplification and also to predict the response to trastuzumab therapy [36]. Some of the earlier studies also show that in terms of the quality of results RT-qPCR may outperform IHC/FISH [37, 38]. At the same time, it is also important to discuss some of the studies that demonstrated discordance of RT-qPCR with IHC/

FISH. Gupta et al. performed studies using 63 patients and found that the results obtained using RT-qPCR and IHC/immunofluorescence were not correlating [31]. Another recent study showed that RT-qPCR may not be a reliable technique for evaluating the *HER2* amplification [30]. Even in the scenario that a few studies show discordance of RT-qPCR results with that of IHC/FISH, most of them stress the need for reliable quantitative techniques to assess *HER2* amplification, especially when the IHC results are ambiguous.

Although our study showed that the larger tumors had higher *HER2* scores (Figure 3), compared to the smaller ones, there was some variability in the data, which might be attributed to the inherent complexity and hetergoneity of cancer [39]. Other factors such as genetics, environment, and other molecular features, can influence *HER2* expression, and this warrants further research, since this could be significant for personalizing the treatment approach. It is a well-established fact that HER2 amplification is often associated with aggressive tumor growth and poor prognosis [40]. In our samples, the majority of tumours with a high *HER2* score (3+) also had a high SBR grade (grade 3), which is based on three histological features: tubular differentiation, nuclear pleomorphism, and mitotic proliferation index (Figure 4). A high SBR score generally indicates more aggressive tumor behavior and an increased risk of recurrence [41]. Although not linear, the association suggests that tumours with *HER2* overexpression may also have more aggressive histological features, which is crucial for therapeutic decision-making, particularly in identifying patients with optimal response to anti-*HER2* targeted therapy.

In conclusion, the current study shows that one-step RT-qPCR is an accurate, reliable, and fast technique for quantifying *HER2* mRNA. Moreover, compared to the gold standard techniques, it is less expensive, rapid, and offers reproducibility of results. Current studies indicate that the therapy strategy should not be solely designed based on IHC results, under circumstances that IHC offers only a limited dynamic range of analysis. Apart from designing the therapy, quantitative *HER2* expression is significant in tracking the progress of therapeutic response. Our results are in conjunction with several other recently published studies which show that RT-qPCR is a reliable technique and may be easily adapted to all clinical settings. However, further studies at multiple locations are warranted in this direction, recruiting patients with different levels of *HER2* amplification, and at different stages of the disease. It should be noted that the quality of mRNA and the choice of reference genes play a significant role in the results and as laid out by previous studies, the FFPE samples should contain more than 50% of tumor cells for reliable results. Taken together, our studies shed light to the significance of a more standardized quantification of *HER2* expression for better treatment orientation.

Author Contribution Statement

BA carried out most of the experiments. HE helped in performing experiments. HE, AM participated in the design of the study, performed the statistical analysis and helped in the drafted the manuscript. BY participated in the design of the study and were involved with revising the manuscript critically. MK and AB provided and managed the patient's samples and revised the manuscript critically and conducted the external validation of the test. AM conceived, designed, coordinated the study and drafted the paper. All authors read and approved the final manuscript.

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Ethical approval for the use of patient's samples has been obtained from the Casablanca medical school ethic committee

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Any conflict of interest

The authors declare that they have no competing interests.

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