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Advanced breast cancer diagnosis: Multiplex RT-qPCR for precise typing and angiogenesis profiling

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ABSTRACT

Breast cancer (BC) remains the foremost cause of cancer-related mortality, with an estimated 2.3 million new cases anticipated globally. The timely diagnosis of BC is pivotal for effective treatment. Currently, BC diagnosis predominantly relies on Immunohistochemistry (IHC), a method known for its sluggishness, expense, and dependence on proficient pathologists for confident cancer typing. In this study, we introduce a novel approach to enhance the accuracy, speed, and cost-effectiveness of BC diagnosis. We employ multiplex Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) with touch-down methods, which consistently yield significantly lower Cycle Threshold (CT) values. The study evaluates gene expression profiles of HER2, PGR, ESR, and Ki67 genes across 61 samples representing four BC subtypes, using RPL13A as the endogenous control gene. The results demonstrate that our method offers remarkable precision, nearly equivalent to IHC, in detecting gene expressions vital for BC diagnosis and subtyping. Moreover, we explore the gene expression of Hif1A, ANG, and VEGFR genes involved in angiogenesis, shedding light on the metastatic potential of the tested BC tumours. Notably, numerous samples exhibit elevated levels of Hif1A and VEGFR, indicating their potential as valuable biomarkers for assessing metastatic status. Collectively, our RT-qPCR methodology emerges as a powerful diagnostic tool for swiftly identifying BC subtypes and can be complemented with other essential tumorigenic biomarker assessments, such as angiogenesis, to further refine cancer characterisation and inform personalised therapeutic strategies for BC patients. This innovation holds the promise of revolutionising BC diagnosis and treatment, offering expedited and reliable insights for improved patient care.

1. Introduction

Breast cancer (BC) is a heterogeneous disease that is caused by genetic and environmental factors, comprising many different entities and having different biological features and clinical behaviours [1]. A malignancy originating in BC can develop in various breast tissues, including milk-producing glands, lobules, and ducts. It can also start in fatty and fibrous tissues. Classified as non-invasive (in situ carcinoma) or invasive based on tumour growth beyond the basal membrane[2]. IHC is the gold standard in the clinic for measuring HER2, ER, PR, and occasionally Ki-67 protein expression [3]. When HER2 IHC results are ambiguous, (FISH) is frequently used to determine ERBB2 copy number status [3]. While mRNA is used to assess these same markers as part of the widely used Oncotype-Dx test, it is not generally utilized as the basis for treatment regimen selection, and the ASCO/CAP recommendations do not support it [4]. One explanation for this is that IHC tests' in situ value is considered to reduce false positive tests caused by non-tumour tissue contaminating mRNA expression for these important markers [3]. HER2, ER, PR, and Ki-67 were immune-stained using specific antibodies. Receptor-positive nuclei were defined based on ER and PR positivity exceeding 1 %, adhering to ASCO standards [5], and HER2 was graded on a scale of 0–3 [6]. In the end, a score of 3 or more was deemed excessively positive. For HER2 amplification, fluorescence in situ hybridization was not used [7].

The quantitative real-time polymerase chain reaction (RT-qPCR) is a popular tool for identifying genes that influence cancer prognosis and treatment [8]. Following reverse transcription, RT-qPCR is a fast, accurate, and reliable method for quantifying DNA and mRNA [9]. Data normalization is crucial in RT-qPCR due to non-biological variations like changes in cellular input and RNA quality between samples, ensuring accurate gene expression assessments[10]. These issues might be solved by employing a stable reference gene to normalize gene expression

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measurements [11]. The expression of common reference genes (formerly known as housekeeping genes) like ACTB and GAPDH varies, nevertheless, depending on the type of cell [12]. These reference genes are essential for the maintenance of basic cellular activities and are expressed constitutively [13]. The endogenous reference gene is assumed to be an ideal, sufficiently abundant gene with steady expression across many tissues and cell lines under various experimental settings [14]. Absolute and relative/comparative quantification are the two main RT-qPCR quantification techniques that have been developed and are often utilized [15]. Absolute quantification in RT-qPCR relies on a standard curve, using fluorescence signals from dilutions of known samples. This approach ignores sample variability, focusing solely on the constructed curve [16]. By comparing the expression of the target gene in a sample to the expression of a reference gene, relative quantification overcomes this constraint [17]. Regardless of biological variations and experimental settings, the ideal reference genes employed in an RT-qPCR experiment must have constant expression levels [14,18]. Reference genes are internal reaction regulators with a different sequence from the target. A gene must fulfil many crucial requirements to be considered a trustworthy reference [19]. The molecular subtypes of BC based on gene expression have been independently confirmed [20], and have gained widespread acceptance since their identification in 2000 [21]. Gene expression assays like the original subtyping technique have been created to simplify clinical deployment; the most well-known and widely used is the PAM50 assay [22]. Despite this, it appears that there are only a limited number of big datasets with gene expression-based subtyping available because the majority of epidemiological studies use IHC surrogate subtypes to examine the intrinsic subtypes [23]. In addition to the proliferation marker Ki-67 [24], and/or basal-like markers cytokeratin 5 and 6, a three-marker panel including the ER, PR, and HER2 labeling has also been utilized often [25]. RT-qPCR eliminates the need for expert cytologists, providing objective results independent of subjective interpretations, a distinct advantage over IHC [26]. It is a straightforward, highly sensitive, repeatable, and high-yielding throughput method for confirming gene expression variations and quantifying transcript abundances [27].

This investigation aims to establish a comprehensive RT-qPCR method for accurate diagnosis of BC types to resemble substitute immunohistochemistry methods. In addition, extra characterizations regarding the potential severity of the tumours to survival will be studied and established.

2. Materials and methods

Sample collection: A total of 61 tumours FFPE block samples from different groups of BCs were collected from (Shorsh General Hospital/Histopathology department under *Ethics Approval code UoS-Sci-Bio-0011*), including Luminal type, TN, HER2 positive, and TP subtypes, and 9 benign samples from October 2020 to January 2022. Tumours and benign samples were diagnosed and classified according to the WHO Histological Typing of Breast Tumours by the IHC technique by pathologists and only sample blocks with more than two-thirds and more tumours tissue were selected for the investigation also just tumour tissue areas were cut and used for extraction RNA.

RNA extraction: Following the manufacturer's instructions, total RNA was extracted from FFPE samples using the Quick-DNA/RNATM FFPE Kit (ZYMO RESEARCH Catalog Number R1009). The isolated RNA was then stored at −80 °C. The Nano-Drop One C spectrophotometer (Thermo Fisher Scientific) was used to evaluate the concentration and purity of RNA.

Oligonucleotides (Primers and probes): Primers and probes were ordered from (Sigma-Aldrich) company (order number: 8817274745) in a lyophilized form and each was resuspended in PCR-grade water according to company protocol instructions to (100 μM) as the final concentration. The dilution 1:10 was prepared to get 10 μM (Table S1).

2.1. Real-Time Quantitative PCR (RT-qPCR)

In our study, we employed multiplex RT-qPCR (Table 1) to test target gene expression (ESR, PGR, HER-2, Ki67, HIF1A, ANG, VEGF) using specific primers and dual hybridization probes (Fig. 1). The experiments were conducted on a MIC PCR machine (Australia S/N: M0000328) with reference genes (GAPDH and RPL13A). A multiplexing strategy utilizing three reaction tubes per sample was employed. The first tube assessed the expression of (RPL13A reference gene, ESR, PGR, HER2), the second tube measured (Ki67 and RPL13A), and the third tube detected (RPL13A, HIF1A, ANG, and VEGF). The amplification efficiencies (E%) were calculated for each pair of primers and showed that RPL13A: 99.913, ESR: 99.251, PGR: 101.78, HER2: 99.046, Ki67: 98.435, HIF1A: 98.841, VEGF: 102.695, ANG: 100.923, and GAPDH: 109.176 (Table S1), using an equation of calculation ($E = -1+10^{(-1/slope)}$) [28].

2.2. Data analysis

The experiment involved determining the CT values of each gene. The reference genes were used in the computation of ΔCT values. The ΔCT values of target genes were calculated and then inverted by subtracting the maximum PCR cycle number from the value of ΔCT. Then the inverted ΔCT values were normalized mathematically [29,30] to the scale of IHC accordingly, enabling a direct comparison with results from IHC investigations.

The ΔΔCT method was employed to calculate gene expression fold changes. ΔΔCT values were derived from treated samples compared to control samples, determining fold changes using the formula 2ˆ(-ΔΔCT). This technique enabled rapid and precise evaluation of gene expression alterations in response to experimental conditions[31,32].

2.3. Statistical data analysis

To determine the significance of the differences between the benign and malignant data sets, we used GraphPad Prism 8.3.0 software to perform *t*-test statistical analysis in this investigation. Since the *t*-test is a commonly used technique to compare means between two groups, it is especially appropriate for our research.

3. Results

3.1. Reference gene selection and RT-qPCR Protocol optimization

To distinguish an optimal reference gene, we compared the performance of GAPDH and RPL13A with three different PCR programs (Table 2). Luminal type and TP samples were used. The precisely designed primers, along with the choice of RPL13A as the reference gene, ensured the reliability and robustness of our experimental results, the M (stability value) of GAPDH was 1.686 while for RPL13A was 0.288 in our experiments, and based on previous experiments on reference gene selections methods for RT-qPCR [33].

3.2. Expression of (ESR, PGR, HER2, and Ki67) genes in benign samples

A pathologist physician made a diagnosis of benign breast tumours. This investigation aims to demonstrate the RT-qPCR method's complete accuracy in identifying these samples as benign. In particular, it accurately classified these samples as positive for ESR and PGR but negative for HER2 (Table S7).

Table 1
Touch-down RT-qPCR protocol.

	cDNA formation	Initial denaturation	Pre-Cycle							Cycling 40 cycles	
			3 cycles		3 cycles		3 cycles				
Temperature	50 °C	95 °C	95 °C	70 °C	95 °C	67 °C	95 °C	63 °C	60 °C	95 °C	60 °C
Time	10 min.	2 min.	10 s.	15 s.	10 s.	15 s.	10 s.	15 s.	15 s.	5 s.	30 s with Data collection

Table 2
Reference gene expression optimization using different RT-qPCR programs.

Reference gene	STD PCR	STD PCR with separate annealing (58 C) and extension steps.	Touch Down PCR
GAPDH	32.81	30.28	23.9
GAPDH	32.45	30.61	23.62
GAPDH	33.15	NA	NA
GAPDH	33.01	NA	NA
NTC GAPDH	–	–	–
RPL13A	27.53	27.88	19.57
RPL13A	29.06	27.48	19.92
RPL13A	27.01	NA	NA
RPL13A	27.17	NA	NA
NTC RPL13A	–	–	–

Abbreviations: GAPDH: Glyceraldehyde-3-phosphate dehydrogenase, RPL13A: Ribosomal protein L13a, NA: Not available, NTC: Negative template control, CT: cycle threshold, STD: Standard.

3.3. Comparative BC biomarker gene expression in RT-qPCR and IHC methods

We evaluated the accuracy and reliability of multiplex RT-qPCR for BC diagnosis by comparing it with standard IHC methods on the same samples. Diagnostic compatibility was assessed based on qualitative agreement (Yes/No) between positive and negative results.

3.3.1. HER2 gene expression in different types of BC

Investigation of HER2 diagnosis in various BC types via RT-qPCR (Table 3), followed by comparison with IHC method." HER2 gene expression is first measured by RT-qPCR, which offers molecular understanding. The outcomes are then compared to those of IHC: which highlights the presence of the HER2 protein in tissue samples to help identify certain indicators. Fig. 2D: illustrates HER2 gene expression in four types of BC. Samples (L2, L5, and L6) were given the results in RT-qPCR but not in IHC, and vice versa to sample (L48), the diagnostic compatibility was (29/33) between RT-qPCR/IHC techniques in the Luminal type of BC (Table S2). In TNBC the match compatibility between the two methods is 100 %, which means all samples were not expressed HER2, RT-qPCR/IHC matching scores were (12/12) (Table S3). In HER2 overexpression BC types, the match compatibility between the two methods is 100 %, which means that samples expressed HER2, RT-qPCR/IHC matching scores were (10/10) (Table S4). Finally, the match compatibility between the two methods is 100 % in the TP type of BC, all samples were expressed HER2 in TP type. RT-qPCR/IHC matching scores were (6/6) (Table S5).

3.3.2. PGR expression in the different types of BC

Investigating PGR gene expression in various kinds of BC using RT-qPCR (Table 3), and comparison to the IHC method." In this study, RT-qPCR is used to measure the PGR gene expression levels in several BC types. Fig. 2C: illustrates PGR gene expression in four types of BC. In the Luminal type the match compatibility between the two methods is 100 % means all samples were expressed PGR, and RT-qPCR/IHC matching scores were (33/33) (Table S2). Also in TNBC the match compatibility between the two methods is 100 %. All samples were not expressed PGR, and RT-qPCR/IHC matching scores were (12/12) (Table S3). But some variations found in the HER2 overexpression type, and the match compatibility between the two methods is 90 %., samples were matched the expression of the PGR gene in HER2 type BC except (40 HER2), and the sample RT-qPCR/IHC matching score was (9/10) (Table S4). Finally, the match compatibility between the two methods is 100 % in TP type, which means all samples were expressed PGR and RT-qPCR/IHC matching scores were (10/10) (Table S5).

3.3.3. ESR gene expression detection in the BC types

Studying the state of the ESR gene expression across several BC categories is the goal of the study. To begin with, RT-qPCR measures ESR gene expression and offers molecular insights. These results are then compared using IHC. Fig. 2B: illustrates ESR gene expression in four types of BC (Table 3). Luminal type BC shows the match compatibility between the two methods is 93.93 %, all samples were expressed ESR except (L32 and L37) which means RT-qPCR/IHC matching scores were (31/33) (Table S2). While in TNBC, the match compatibility between the two methods is 100 %, All samples were not expressed PGR, RT-qPCR/IHC matching scores were (12/12) (Table S3). However, in the

Table 3
RT-qPCR method accuracy per single gene in each BC type.

Sample BC types	Number of samples	RT-qPCR/IHC matching score for each gene			
		ESR	PGR	HER2	Ki67
Luminal type	33	31/33	33/33	29/33	26/27 6 Samples in IHC were not assessed for Ki67
HER2 Overexpression	10	9/10	9/10	10/10	7/7 3 Samples in IHC were not assessed for Ki67
TNBC	12	12/12	12/12	12/12	9/10 2 Samples in IHC were not assessed for Ki67
TP	6	5/6	6/6	6/6	5/6 All samples in IHC were assessed for Ki67

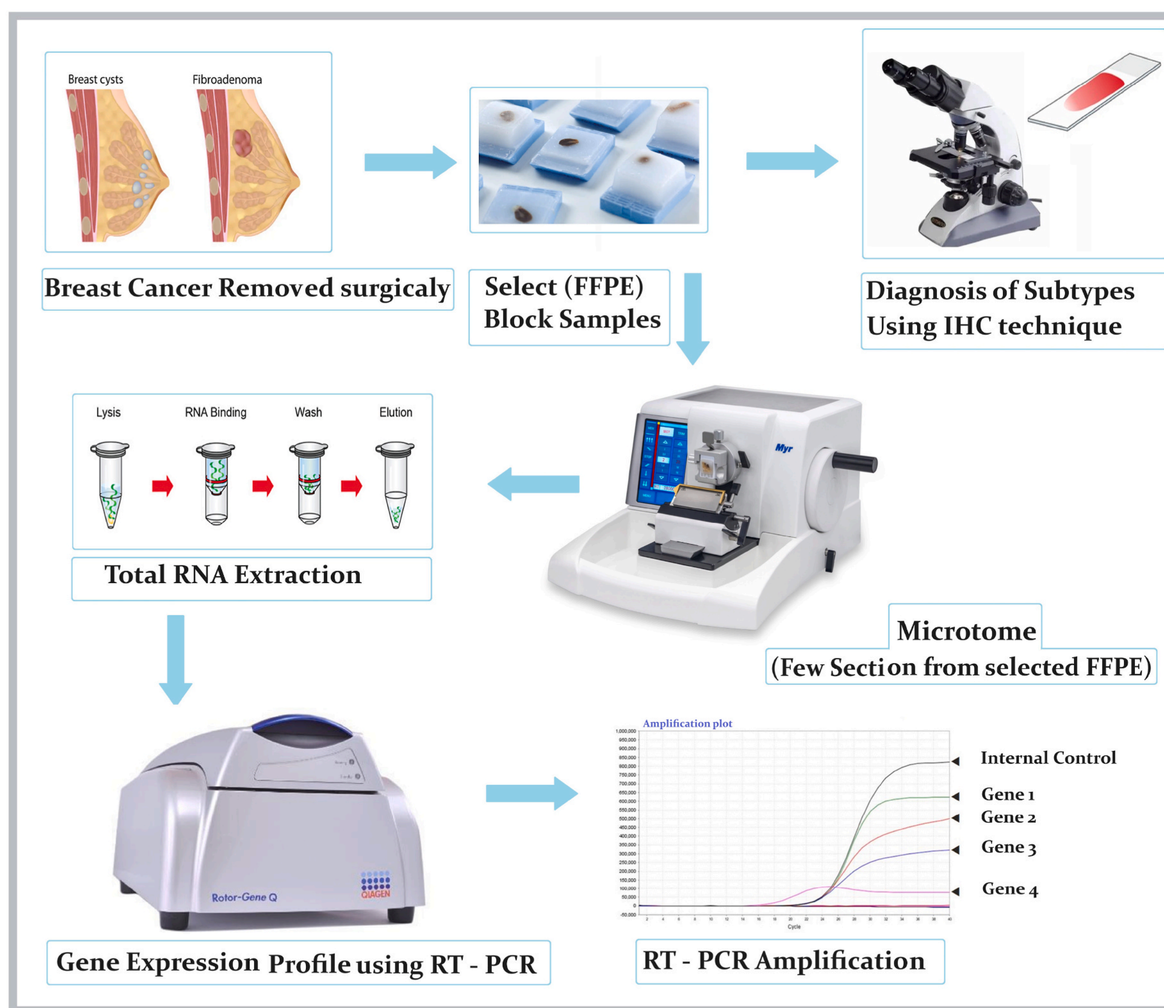

Fig. 1. The graphical illustration that outlines the current study.

HER2 overexpression type of BC, the match compatibility between the two methods is 90 %. Samples were not expressed ESR by RT-qPCR method except sample (10 HER2) which had an IHC score of 2 and was not detected by RT-qPCR. So the sample RT-qPCR/IHC matching score was (9/10) (Table S4). In TP type BC, the match compatibility between the two methods is 83.33 %. All samples were expressed HER2 in TP type and detected by RT-qPCR except (TP 39), RT-qPCR/IHC matching scores were (5/6) (Table S5).

3.3.4. Ki67 gene expression detection in the Luminal group

Expression of Ki67 in the Luminal type of BC shows 96.55 % compatibility between the two methods, RT-qPCR/IHC matching score was (27/28), and the sample (L5) was not given a result in RT-qPCR but had a result in IHC. Samples (L25, L29, L30 L33, L37, and L44) in IHC were not assessed for Ki67 and we could not compare between RT-qPCR and IHC (Table S2). While in TNBC RT-qPCR/IHC matching scores were (9/10) because the sample (TN 19) was not detected by RT-qPCR while having a result in IHC. Samples (TN 16 and TN 17) (Table S3) were not assigned by IHC and could not be compared between them by RT-qPCR and IHC. In HER2 overexpression type of BC show RT-qPCR/IHC matching scores (7/7), samples (8 HER2, 40 HER2, and 59 HER2) (Table S4) were not assigned by IHC and could not be compared between them by RT-qPCR and IHC. However, in the TP type of BC, the match compatibility between the two methods is 83.33 %. All samples were expressed Ki67 in TP type by RT-qPCR except (TP 61) as shown in (Table S5). RT-qPCR/IHC matching scores were (5/6). Fig. 2E: illustrates Ki67 gene expression in four types of BC.

Amplification plots using logarithmic transformed data curve of RT-qPCR of expression genes:

3.4. Diagnosis of BC types using RT-qPCR method in comparison with IHC results

This study uses RT-qPCR to classify BC by measuring the expression levels of the ESR, PGR, HER2, and Ki67 genes. The outputs of Immunohistochemistry (IHC) are then compared to these findings. The objective of the study is to clarify the agreement between RT-qPCR and IHC methods in identifying BC types, adding to our understanding of molecular diagnostics. We observed five samples (L2, L5, L6, L32, and L37) in luminal type, (TP 39), and (40 HER2) in the HER2 overexpression group (Table S2) were not matched. In the present investigation, the positive group or the similar outcome is 84.84 % (28/33) using RT-qPCR/IHC in luminal type BC. The match compatibility between the two methods is 90 % in HER2 overexpression type. But the proportion of the TP group is 83.3 %. sample (TP 39) did not give the result in RT-PCR but had a score in IHC. The diagnosis match compatibility between RT-PCR/IHC was (5/6). While the results of (RT-qPCR and IHC) are the same in the TNBC type, with 100 % compatibility RT-qPCR/IHC matching scores were (12/12).

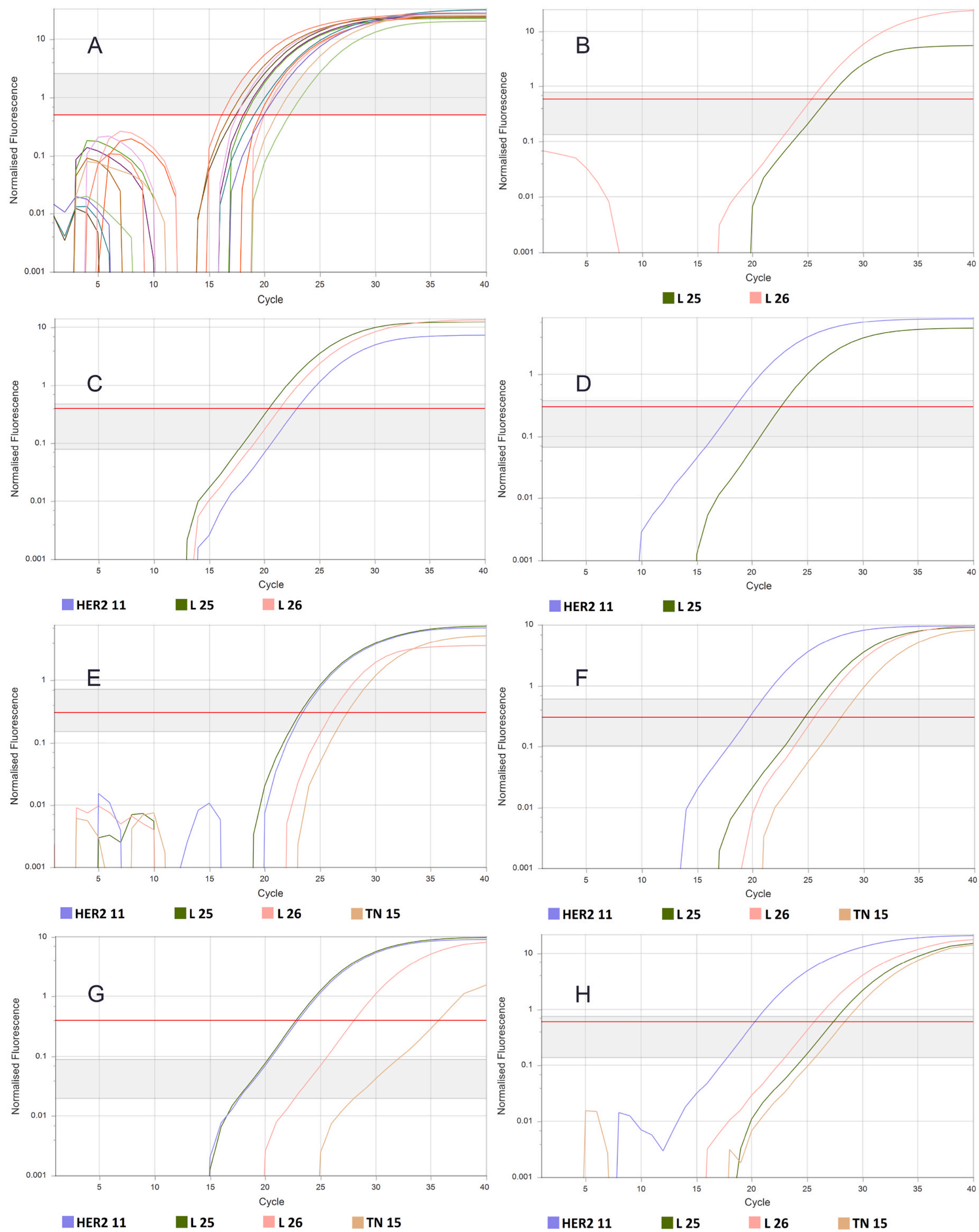

Fig. 2. Shows the Log transformed of amplification plots which demonstrate the expression levels of particular genes, including (A) RPL13A as a reference gene was amplified in all samples as a control, (B) ESR gene expression was only expressed in samples (L25 and L26) but not in (HER2 11 and TN15), (C) PGR gene expression were expressed in samples (HER2 11, L25, and L26) but not in (TN15), (D) HER2 gene expression was expressed in samples (HER2 11 and L25) but not in (L26 and TN15), (E) Ki67 gene expression were expressed in samples (HER2 11, L25, L26, and TN15) which are essentials for determining the subtypes of BC. Angiogenesis gene expressions including (F) HIF1A were expressed in samples (HER2 11, L25, and L26 and TN15), (G) ANG marker in samples (HER2 11, L25, and L26, and TN15), and (H) VEGF marker were expressed in samples (HER2 11, L25, and L26, and TN15).

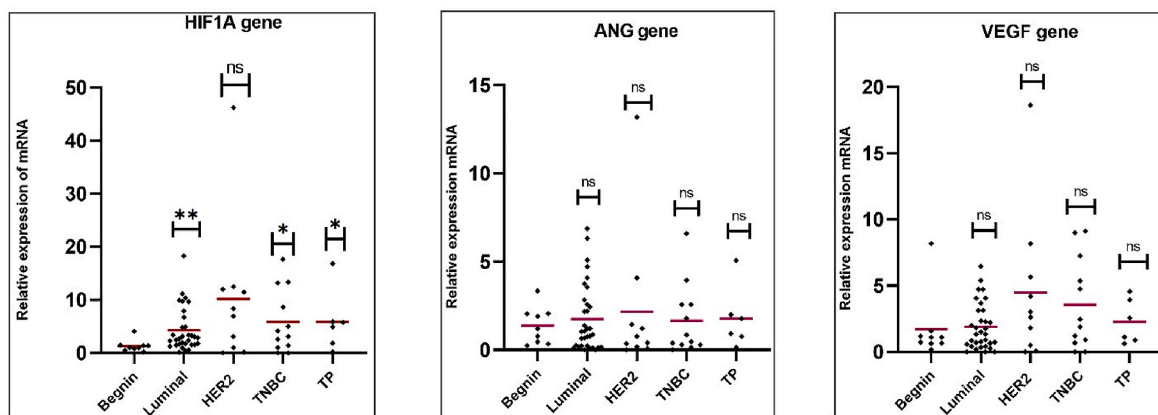

Fig. 3. Scatter plot analysis represents a change in the fold of gene expression that related to angiogenesis (HIF1A, ANG, and VEGF) in four types of BC (Luminal, HER2, TNBC, and TP) that were studied in our experiments located on the X-axis and relative gene expression mRNA on Y-axis, employing the Mann-Whitney *U* test to compare the Mean value of each group with the benign samples based on statistical significance differences in relative mRNA gene expressions * $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns = not significant. By using GraphPad Prism (8.3.0) software.

3.5. Angiogenesis genes as metastasis biomarkers for BC types

In addition to the four biomarkers (ESR, PGR, Her2, Ki67) we added an extra three markers (ANG, HIF1A, and VEGF), gene expression plots shown in (Fig. 3). These extra biomarkers which illustrate the fold of expressions in each gene determined are related to the ability of the tumours to generate new blood vessels, helping them escape into the blood circulations toward the metastatic step.

Scatter plot of gene expression fold of change for Angiogenesis biomarkers (HIF1A, ANG, and VEGF) genes in four different groups of BC samples shown in Fig. 3 incorporated with Mann-Whitney *U* test.

3.6. Statistical analysis

We analyzed benign and malignant tumour data, employing the Mann-Whitney *U* test as a non-parametric alternative to the *t*-test. Comparing gene expression levels of (ESR, PGR, HER2, and Ki67) between BC subtypes using RT-qPCR and IHC techniques, our study ensured accuracy and reliability through precise computations facilitated by GraphPad Prism 8.3.0 software (Fig. 4). This robust statistical approach enabled us to identify significant differences, enhancing the integrity of our results in characterizing BC subtypes.

In the analysis of gene expression, the ESR and PGR genes exhibit highly significant differences between the "IHC" and "RT-qPCR" groups, as indicated by extremely low p-values (<0.0001). Specifically, the median values are notably higher in the "IHC" group than in the "RT-qPCR" group for both genes. Conversely, for the HER2 gene, the p-value (0.8359) suggests no substantial difference between the groups, failing to reject the null hypothesis. In contrast, the Ki67 gene displays a significant difference (p-value = 0.0033) between the groups, indicating varied expression levels. The "RT-qPCR" group exhibits a significantly higher median value than the "IHC" group, providing strong evidence of differentiation in Ki67 levels. These findings underscore the efficacy of RT-qPCR in distinguishing gene expression patterns, especially for ESR, PGR, and Ki67 genes, compared to IHC methods.

4. Discussion

The conventional method for BC-type diagnosis, is (IHC), which involves staining prominent biomarkers like Estrogen receptor, Progesterone receptor, HER2, and Ki-67 proteins in histological samples. While reliable, IHC is time-consuming and demands skilled pathologists. This study proposes a faster and more precise alternative: quantitative Real-time PCR (RT-qPCR). This method, completed within 4–5 h, offers a quicker diagnosis, eliminating the need for extended laboratory time. Our approach focuses on the standard 4 biomarkers used in IHC, making it a straightforward, reliable diagnostic tool for BC [34]. Here, we tried both a qualitative and relatively quantitative approach to get nearest to the IHC method that uses grading system scores for each biomarker to reach a consistent diagnosis. Selecting an appropriate PCR protocol is essential for precise gene expression analyses. Although the MamaTyper commercial kit used the same biomarkers for the diagnosis of BC types mentioned in our work [35] except that we used a different reference gene (RPL13A) MamaTyper is use beta-2-microglobulin and calmodulin 2 and also different PCR protocol that was optimal to our work. Another comparison is that we used the same scale in normalized data RT-qPCR to direct comparisons with IHC data. Touch-down PCR turned out to be a very successful approach that involved progressively lowering the annealing temperature. It reduced CT values compared to the usual PCR technique by a minimum of 7 CT [36]. Furthermore, the touch-down PCR technique significantly improved method specificity in addition to aligning gene amplification. The next step in establishing a successful RT-qPCR protocol is to carefully choose the proper reference gene. To this end, finding cell-specific control genes is recommended before conducting RT-qPCR experiments. [12] detected that RPL13A was the most stable gene in the consecutive passages of SK-BR-3. As a result, it's critical to verify reference genes before utilizing them in a study, because employing a non-validated reference gene might lead to erroneous conclusions based on faulty data [37]. Investigation in humans derived from abdominal fat liposuction discovered that RPL13A was the best reference gene [38].

To be able to properly evaluate the accuracy of RT-qPCR in diagnosing BC, and to take sample variances into account, ΔCT is computed. The ratio of mRNA to protein is linear even though it isn't exactly (1:1) but linear. Increased production of proteins results from higher mRNA levels, which open the door to post-transcriptional and post-translational modifications. To put both RT-qPCR and IHC data on the same mathematical scale and to make meaningful comparisons, we first invert the ΔCT value, which represents gene expression inversely, greater expressiveness is indicated by lower values in PCR. Lower values in the linear IHC data indicate lower quantities of protein. After that, we align both measurements by normalizing the ΔCT value to IHC scales and inverting it. We argue here, that this method is the first time to be used to compare RT-qPCR with IHC data on the same scale. We performed multiplex, multi-colour PCR reactions where HER2, ESR, PGR, and RPL13A as reference genes were all measured in one tube, and Ki67 with RPL13A in a second tube. For the HER2 gene, out of 61 samples, only 4 samples are in absolute disagreement. Therefore, in 7.02 % of the tests, the results can be considered false positive. This makes the accuracy of the RT-qPCR test to correctly identify HER2 expression (as Yes

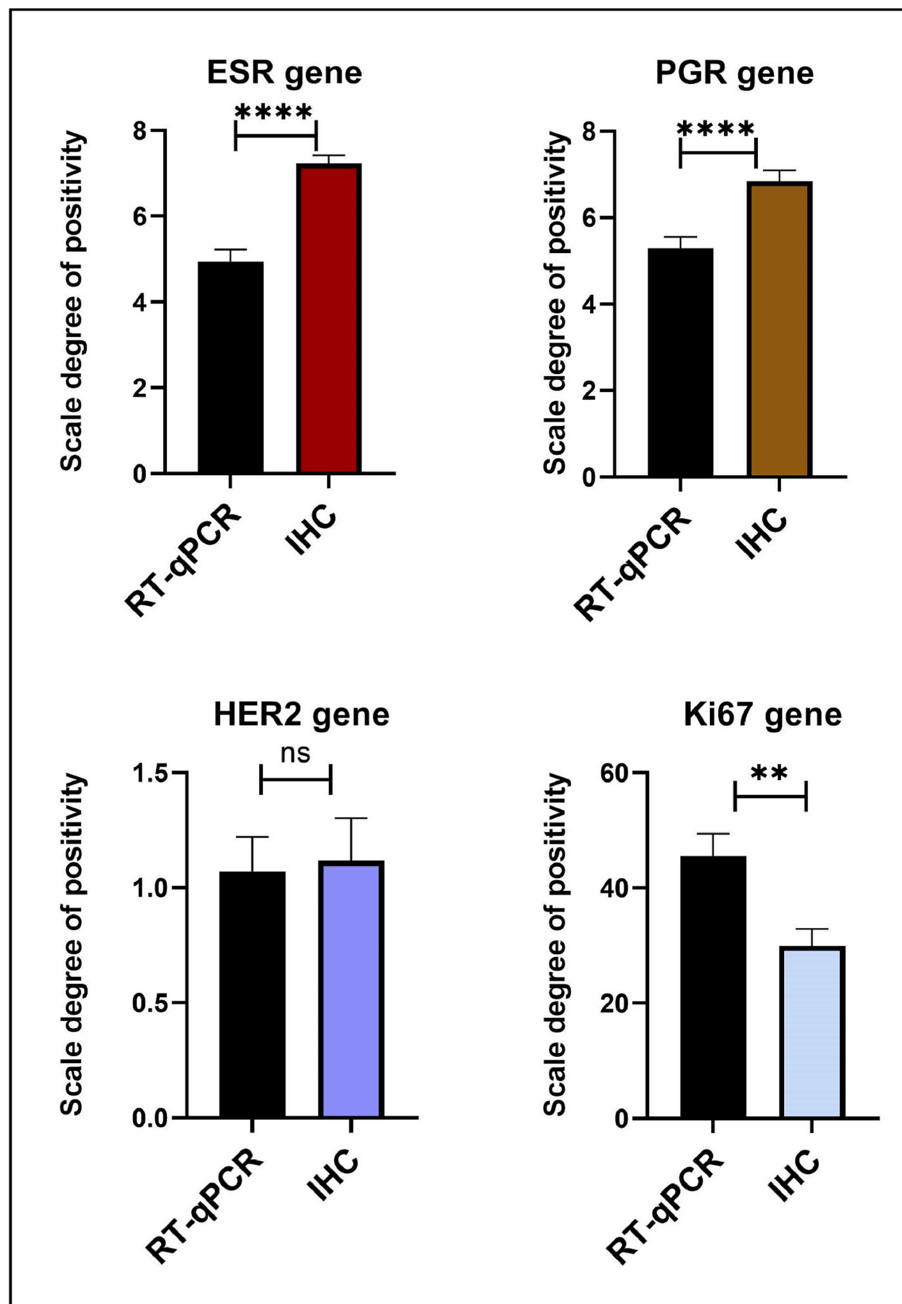

Fig. 4. Bar chart of statistical analysis between two different techniques used in BC-type diagnosis. Results were reported comparing the two techniques based on statistical significance * P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant.

vs. No expression) approximately 93 % accurate in the Luminal BC group. Interestingly the RT-qPCR method for HER2 gene expression in all other BC groups, namely; TNBC, TP, and HER2 group (HER2 overexpression) the method performance is 100 % accurate.

Except for Luminal BC, most BC subtypes exhibit good accuracy in HER2 evaluation using both RT-qPCR and IHC. Luminal B subtypes might occasionally show HER2 amplification or overexpression, which justifies a HER2-positive classification [39]. HER2 grading in luminal BC is based on the level of HER2 amplification or overexpression, according to ASCO/CAP criteria using both FISH and IHC. Potential technical and human error inconsistencies, such as problems with tissue fixation, inconsistent staining, and subjective pathologist interpretation, should be acknowledged.

Nearly complete agreement exists between the PGR expression results for the various BC groups, which substantially validates the accuracy of RT-qPCR. Within the HER2 over-expression group, there is a single exception in which an IHC grade of 3 from (1–8) did not result in a signal detected by RT-qPCR. Even while overall accuracy is very high, the differences need to be taken seriously, particularly when it comes to luminal samples with high positivity.

High levels of Ki67 in BC cells are generally associated with a more aggressive tumour phenotype, higher tumour grade, and worse prognosis [40]. Although some samples are missing Ki67 results in IHC, we performed RT-qPCR for all samples, These missing data cause the statistical significance of our comparisons less significant, non the less we can consider our results highly significant. Overall after seeing all genes with diagnostic features, we found our method's accuracy is between 83 and 100 %, with TP being less predictive, but the fact that we only have 6 samples, it hard to make an accurate estimation. Luminal BC on another hand, makes a better estimation with 33 samples and 84 %

accuracy, followed by the HER2 group at 90 % and Triple negative at 100 % accuracy.

Except for the luminal subtype, all three primary genes have exceptional HER2 gene accuracy, reaching 100 % in all forms of BC. This disparity in the luminal subtype is ascribed to HER2 mRNA levels that can be found by RT-qPCR, which are not yet reflected at the protein level by IHC. Lower true positive results are closely mirrored by normalized RT-qPCR values, indicating a propensity for false positives rather than false negatives. False negatives would indicate a failure to detect significant HER2 mRNA levels, which could affect the method's accuracy in detecting BC. False positives could be caused by trace mRNA copies in the sample. As for PGR and ESR, the low mismatches that we detected in different samples (PGR gene: 40-HER2; ESR gene: L32, L37; and 10 HER2), it's the other way around, RT-qPCR could not detect any signal while there are significant high IHC degrees in the BC types, meaning RT-qPCR is giving false negative results. To this end, we can confidently mention here, that the RT-qPCR method is highly reliable and offers a great deal of feasibility, and we hope to further improve it to be considered for proposed BC diagnostic purposes substituting the costly and time-consuming IHC method.

In this scientific work, we also wanted to add another layer of information to our diagnostic approach, and this was done by adding three other genes as multiplex RT-qPCR methods for all the samples used. We choose Hif1A, ANG, and VEGF gene expression as potential diagnostic or prognostic features of the tests. These three genes are mainly responsible for (Angiogenesis) [41], where Hif1A senses the hypoxia state of the tumour and leads to the expression of ANG and VEGF genes that regulate blood vessel formation in the next steps [42]. Through the results obtained, we can identify that Hif1A is significantly higher expressed than the other genes in almost all the samples. It can be noted in Luminal samples only 5 samples out of 33 show 10 to 15-fold expression compared to other genes, but in TN and HER2, most of the samples show high expression of Hif1A. The amount of HIF1A in cells and the level of oxygen are tightly connected. Therefore, reducing HIF1's expression or activity could prevent the formation of tumours [43]. VEGFA is a crucial modulator of both healthy and pathological angiogenesis and is a confirmed target for anti-angiogenesis therapy in the clinic [44].

We showed that VEGF is relatively higher expressed than ANG (Figuers.3.3 to 3.6) in the majority of the samples, which could be explained by the biology of ANG gene expression as ANG is considered to have its own RNase activity that may negatively regulate its expression [45]. We showed that in the case of BC, Hif1A is the highest expressed gene, thus a therapeutic approach to inhibit this molecule offers a promising target in BC therapy.

To use the angiogenesis gene expression data in combination with diagnostic genes, a much higher number of samples are needed in all the different types of BC, We need to have more data regarding the patient diagnostics and follow-up procedures.

5. Conclusions

Touch-down PCR is a powerful tool for reducing the CT value in RT-qPCR compared to standard PCR methods. Also, RPL13A is the best reference gene used in gene expression detection due to its stability in all types of BC. Multiplex RT-qPCR offers a fast, reliable, and cost-effective method for the diagnosis of some types of BC. In addition to basic diagnosis, it is possible to use other genetic expression markers like angiogenesis to further characterize cancer and potentially identify the risk of metastasis much earlier than the traditional method, thus offering a better chance to treat cancer effectively.

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Statement from the institutional review board and informed consent statement

The study was conducted by the Declaration of Helsinki, and approved by the Ethics Committee at the University of Sulaimani (Approval code UoS-Sci-Bio-0011).

The accessibility of the data documents statement

The data that support the findings of this study are available from the author (HA) on request.

CRediT authorship contribution statement

Harem Abdalla Awrahman: Writing – original draft, Validation, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Dlnya Mohamad:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101615.

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