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Diagnostic ability of real-time quantitative polymerase chain reaction versus immunohistochemistry for Ki-67 assessment in breast cancer: An Indian perspective

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***Background & objectives*: Breast cancer is the most common cancer of women. Inferior prognosis in some patients has been attributed to the higher proliferative capability of the tumour. Immunohistochemistry (IHC) for Ki-67, despite being a simple and cost-effective method, has not become a valid tool to evaluate this biomarker. This is ascribed to variation in pre-analytical and analytical techniques, variable expression, hotspot distribution and inter-and intra-observer inconsistency. This study was aimed at defining the analytical and clinical validity of real-time quantitative polymerase chain reaction (RT-qPCR) as an alternative to IHC evaluation.**

***Methods*: This study included a total of 109 patients with invasive breast cancers. Ki-67 IHC visual assessment was compared with the mRNA value determined by RT-qPCR. Concordance between both the methods was assessed. Receiver operating characteristic (ROC) curve analysis and Cohen's kappa value with intraclass correlation were performed.**

***Results*: The threshold value for Ki-67 by RT-qPCR obtained by ROC curve was 22.23 per cent, which was used to divide breast cancer cases into high proliferative and low proliferative groups. A significant correlation was observed between both the breast cancer groups formed using RT-qPCR threshold as well as median laboratory value of Ki-67 labelling index by IHC.**

***Interpretation & conclusions*: The study results showed a significant correlation between the two methods. While IHC is subject to technical and interpretative variability, RT-qPCR may offer a more objective alternative.**

Key words Breast cancer - immunohistochemistry - Ki-67 - real-time quantitative polymerase chain reaction

Breast cancer is the most common cancer in women worldwide, accounting for 23 per cent of all cancer cases[1]. Breast cancer treatment has evolved immensely following widespread use of predictive biomarkers such as hormone receptor (HR) and human epidermal growth factor receptor 2 (HER 2). Inferior prognosis in some cases which do not respond to the treatment, is dependent on the proliferative capability of the tumour.

This attribute of the tumour has been used in guiding treatment in clinical practice[2].

Immunohistochemistry (IHC) for proliferation marker Ki-67, despite its acknowledged utility, simplicity of technique and easy interpretability, has not become a valid tool to evaluate this biomarker. Ki-67 is a nuclear protein that is present at variable intensity throughout the cell cycle, except G0 phase. While weak in G1 phase, its intensity increases further as the cell cycle progresses with heavy perinucleolar condensation in G2 and S phases followed by parachromosomal concentration during mitosis[3]. The Ki-67 activity is also not uniform and is focally crowded at hotspots. Variability of pre-analytical and analytical practices, variable protein expression during different phases especially G1 phase and hotspot distribution are the other reasons for the failure of IHC for Ki-67 not occupying a vantage position that it so amply deserves. The weak expression in G1 may not evoke strong enough staining to be clearly viewable, and the hotspots may either not be represented in biopsy or variably included[4]. Ki-67 counting may be done in hotspots or assessed by the average percentage staining over the entire section. Which one of these methods translates into a better representation of tumour biology is a question that remains unanswered for breast cancers. This is in contrast to neuroendocrine neoplasms where hotspot counting of 2000 cells has been made the standard[5]. Pre-analytical variations of fixatives, time of fixation and choice of fixative used along with analytical variables such as method of antigen retrieval, selecting the optimal clone among antibodies, diverse staining platforms and signal generation systems further add to confounding results.

This is brought to the fore in a large meta-analysis of Ki-67 expression in 32,825 patients of breast cancer which surmised association of high Ki-67 with worse survival, but also stated that 'marker is not ready for routine use'[6]. Another retrospective analysis of Ki-67 concluded that there was no optimal cut-off point despite its unquestionable role as a prognostic parameter in breast cancer patients[7]. The American Society of Clinical Oncology Tumor Marker guidelines do not recommend routine Ki-67 assay[7], the primary reason being poor interlaboratory comparability and lack of standard operating procedure (SOP). The guidelines, however, do not question the value of the Ki-67 evaluation. The international Ki-67 in Breast Cancer Working Group have published their recommendations concerning the evaluation and interpretation of Ki-67 to enhance interlaboratory comparability and hence the analytical validity. They emphasized its great value in prognosis, predicting response to treatment and as a dynamic marker of treatment effectiveness[7]. Another meta-analysis involving 12,155 patients exhibited high Ki-67 positivity to confer greater risk of recurrence and worse survival[8]. While the value of Ki-67 has been amply established, its best method of assessment and cut-off point to discriminate risk categories is not well established. The thresholds for good and bad prognosis obtained in different research studies are summarised in Table I[9-14].

Using real-time quantitative polymerase chain reaction (RT-qPCR) to quantify gene expression using mRNA has potential advantages over IHC by being more objective and quantitative, thus reducing bias[15]. Several researchers have compared RT-qPCR and IHC for evaluating predictive breast markers[16-18]. Many among these have found the RT-qPCR as a superior technique in determining tumour growth fraction in breast cancers[15,19,20]. Moreover, the expensive multigene risk profilers use gene expression quantification on expression array or by RT-qPCR and allocate heavy weightage to this expression. This study having ensured that all modifiable pre-analytical and analytical variables of IHC were controlled, was conducted to compare and correlate the result of IHC with those

Table I. Ki-67 labelling index (LI) cut-offs determined for breast cancer by various research groups

Research groups	Ki-67 LI cut-off (%) (low-risk vs. high-risk)	Breast cancer subgroups
Saint Gallen meeting 2011[9]	14	High and low proliferation groups
Saint Gallen conference 2013[10]	20	High and low LI
Saint Gallen conference 2015[11]	Median value of laboratory	High and low LI
Petrelli *et al*[12]	25	High and low-risk of death
Ohara *et al*[13]	20	Short and long RFS
Bustreo *et al*[14]	20	High and low-risk

RFS, recurrence-free survival

obtained by RT-qPCR to define the analytical validity of RT-qPCR as an alternative to IHC evaluation of Ki-67 in breast cancer.

Material & Methods

This prospective study was conducted in a tertiary cancer care centre of north India (Rajiv Gandhi Cancer Institute & Research Centre, Delhi) involving 109 patients with invasive breast carcinoma (no special type) from September 2016 to July 2017. The study was restricted to cases which were biopsied before any form of preoperative therapeutic intervention was done. This was to ensure that optimal time to fixation and time of fixation and other pre-analytical variables were controlled. Newly diagnosed and previously untreated consecutive patients of all age groups and both genders were included. The study was approved by the Institutional Review Board and Ethics Committee, and written informed consent was obtained from all patients.

A sample size of 100 was calculated assuming that area under curve (AUC) of 0.70 for RT-qPCR was significant from the null hypothesis value of 0.5 and expecting to include the same number of negative and positive cases with α-level 0.05 and 95 per cent power. MedCalc 18.6 (MedCalc Software, Belgium) was used for calculating the sample size.

Immunohistochemistry (IHC) protocol: All core biopsies were fixed for 6-48 h in 10 per cent neutral-buffered formalin as per the in-house protocol. Paraffin-embedded blocks were made and the sections (4 µm) were cut. Haematoxylin and eosin-stained sections were examined. Immunostaining for Ki-67 (Mouse monoclonal antibody clone MIB-1, Dako, Glostrup, Denmark A/S) using Ventana BenchMark XT 750-700 Automated IHC/ISH autostainer (Tucson, USA) was performed as per the manufacturer's protocol using heat-induced antigen retrieval at alkaline *p*H of 8.6 in Tris buffer for one hour followed by 32 min of primary antibody incubation at 1:75 (v/v) antibody dilution. The signal amplification and signal generation were accomplished using polymer-based ultraview system by Ventana (Tucson, USA). The Ki-67 percentage score was defined as the percentage of positively stained tumour cells (nuclear staining of any intensity) among the total number of malignant cells assessed in the areas of hotspot activity. Hotspot assessment was done to overcome inadequate tumour sampling and for easier standardization. At least 2000 cells were assessed wherever possible and not less than 500 cells were evaluated in every case. All cases were examined on a Nikon microscope (ECLIPSE E200, Japan). Consensus was worked out whenever the difference in count was >5 per cent. The indeterminate cases were not included in the analysis. The histological grading and risk categories were used as defined by Strand *et al*[21].

Real-time quantitative PCR procedure: RNA from formalin-fixed paraffin-embedded (FFPE) samples was extracted. Macro-dissection of the tumour-rich area was performed. Total RNA was isolated using Promega Reliaprep FFPE Total RNA Miniprep System (USA). RNA concentration was measured using Qubit 3.0 Fluorometer (Life Technology, USA). cDNA was generated using Omniscript RT-PCR Kit (Qiagen, Germany). Ten nanogram of RNA was used for cDNA preparation. Ki-67 mRNA expression levels from total RNA were determined using pre-ordered Thermo TaqMan gene expression assay (Thermo Fisher Scientific, USA). Ki-67 Taqman assay (1 µl of primer probe) was used in 25 µl reaction with Taqman Universal Mastermix (Applied Biosystem, USA). β-actin was taken as the normalizing transcript. PCR was performed on Qiagen RGQ real-time PCR. β-actin forward primer (5'-CCACACTGTGCCCATCTACG-3'), reverse primer (5'-AGGATCTTCATGAGGTAGTCAGTC AG-3') and β-actin probe 5,6 fluorescein (FAM)-5'-ATGCCCTCCCCCATGCCATCCTG-3'-caboxytetramethylrhodamine (TAMRA) were used. RT-qPCR thermocycling protocol was followed as: 50°C for two minutes [uracil-DNA glycosylase (UNG) incubation], 95°C for 10 min (initial denaturation), 94°C for 30 sec (denaturation), and 60°C for 60 sec (anneal and extend) 40 cycles.

Threshold was set at 0.01 for analysis. The normalization was used to avoid the impact variability in the RNA quality and quantity and the variability in the reverse transcription efficiency among samples. The normalized expression (ΔCt) was determined by subtracting the average Ct value for β-actin from the average Ki-67. Relative quantification of qPCR data using ΔΔCt or ΔΔCq method was performed[22] and a percentage was obtained from this value.

Statistical analysis: SPSS statistical package, version 23.0 (SPSS Inc., Chicago, IL, USA) was used for analysis. Statistical summaries were presented as mean ± standard deviation for continuous variables while frequencies and their respective percentages were used to summarize categorical variables. Spearman's

correlation coefficient was used for non-parametric variables as a measure of association and strength of linear relationship between variables. Receiver operating characteristic (ROC) curve analysis was carried out to determine the optimal cut-off value of Ki-67 by RT-qPCR by taking IHC value as gold standard. The Youden's index (J), which is the maximum potential effectiveness of a biomarker, was used to summarize measure of the ROC curve[23]. AUC, sensitivity, specificity, positive predicted value (PPV), negative predicted value (NPV) and 95 per cent confidence interval (CI) were presented to summarize the results. Agreement analysis using Cohen's kappa value and intraclass correlation (ICC)[24] was also performed comparing the cut-offs obtained by RT-qPCR and the median laboratory value of Ki-67 labelling index (LI) to determine the level of agreement.

Results

The study included a total of 109 patients comprising 98.2 per cent (n=107) females and 1.8 per cent (n=2) males. The average age of the patients was 52.2±11.2 ranging from 28 to 82 yr. Majority of the patients (n=67) were of histological grade III (61.5%) type. Low-grade tumours (grade I and II) constituted 38.5 per cent of the total cases (n=42) and were considered together for the risk categories.

Biopsy tissue depleted leaving no cancer area in one case and was therefore, eliminated for assessment of HR status and Ki-67 LI. There were 60.6 per cent (n=66) estrogen receptor (ER)-positive cases, 48.6 per cent (n=53) were progesterone receptor (PR) positive and 23.8 per cent (n=26) had HER 2 overexpression or gene amplification. Twenty three (21.1%) patients were found to be with triple negative breast cancers (TNBC).

The median value obtained was 25 and 22.5 per cent by IHC and RT-qPCR, respectively. Significant association was found between Ki-67 LI on IHC with histological grade ($P<0.05$) and clinical stage ($P<0.05$). Ki-67 mRNA expression by RT-qPCR also showed a significant association with both the histological grade ($P<0.05$) and clinical stage ($P<0.01$). Both the methods yielded significantly lower median values of Ki-67 for grade I and II tumours (13.0% by IHC and 16.4% by RT-qPCR) in comparison to grade III tumours (30% by IHC and 27.9% by RT-qPCR).

The threshold value for Ki-67 by RT-qPCR was obtained by ROC curve analysis with maximum possible sensitivity and specificity. On ROC curve analysis, those with scores ≤ highest cut-off corresponding to 100 per cent sensitivity were in low proliferative group, and those with scores > lowest cut-off corresponding to 100 per cent specificity were all categorized as high proliferative. The threshold Ki-67 cut-off value using Youden's index (J) was 22.23 per cent (sensitivity: 78.2% and specificity: 73.6%). Area under the ROC curve was 0.793 ($P<0.001$) with 95 per cent CI of 0.707-0.878 (Figure). PPV (95% CI) and NPV (95%CI) were observed as 75.4 (65.7-83.1) and 76.5 (65.8-84.6) per cent, respectively (Figure). For the purpose of this study, the cut-off value of 22.23 was used to divide breast cancer cases into high proliferative (>22.23) and low proliferative groups (≤22.23). Sixty seven patients were high proliferative while 41 fell into the low proliferative group.

The median value of Ki-67 LI obtained in this analysis was 25 per cent and was used as a cut-off according to the latest Saint Gallen recommendations[11] to categorize breast cancer cases into high and low LI groups. Fifty five patients were of high LI while 53 were found to be of low LI. A significant correlation was observed between both the breast cancer groups formed using RT-qPCR threshold as well as median Ki-67 LI laboratory value by IHC ($P=0.021$ and <0.001 for the two groups, respectively) (Table II). Cohen's kappa value (0.518) and ICC coefficient (0.593) between the two methodologies showed concordance at their respective cut-offs (Table III).

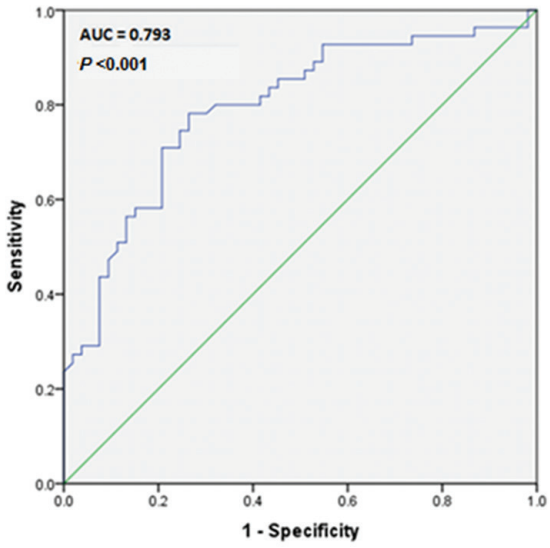

Figure. Receiver operating characteristics curve analysis for prediction of optimum cut-off point of Ki-67 by real-time quantitative polymerase chain reaction.

Table II. Correlation of real-time quantitative polymerase chain reaction for Ki-67 assessment with quantitative immunohistochemistry on various grading systems

Grading system of Ki-67 on IHC	n (%)	Correlation coefficient	*P* value
Saint Gallen classification (2011)[9]			
<14%	41 (37.6)	0.169	0.292
≥14%	67 (62.4)	0.518	<0.001
Saint Gallen classification (2013)[10] and Ohara *et al*[13] recommendations (2016)			
<20%	47 (43.1)	0.268	0.068
≥20%	61 (56.9)	0.492	<0.001
Bustreo *et al*[14], 2016			
<14%	47 (43.1)	0.268	0.068
14-20%			
≥20%	61 (56.9)	0.492	<0.001
Petrelli *et al*[12] recommendations (2015) and median level of Ki-67 labelling index in our laboratory (as per Saint Gallen recommendations of 2015)[11]			
<25%	53 (48.6)	0.317	0.021
≥25%	55 (51.4)	0.477	<0.001

Table III. Agreement determined using Cohen's kappa and intraclass correlation

Agreement	Cut-offs by IHC (%) ≤25 vs. >25	Cut-offs by RT-qPCR (%) ≤22.23 vs. >22.23	Interpretation
Cohen's kappa (*P*)	0.463 (<0.001)	0.518 (<0.001)	Moderate agreement
ICC (95% CI) (*P*)	0.593 (0.41-0.71) (<0.001)		Fairly good agreement

ICC, intraclass correlation coefficient; CI, confidence interval; IHC, immunohistochemistry; RT-qPCR, real-time quantitative polymerase chain reaction

Discussion

In this study an attempt was made to validate and compare the clinical relevance of Ki-67 mRNA expression by RT-qPCR with the Ki-67 LI determined by IHC with utmost control of modifiable variables. Continuous data were dichotomously grouped into high- and low-Ki-67 mRNA categories using the optimal cut-off obtained by ROC curve analysis without compromising upon the sensitivity and specificity. A significant correlation was seen in both the categories recommended by Petrelli *et al*[12], *i.e.*, <25 and ≥25 per cent LI when compared with the two groups of breast cancers formed using the threshold value of RT-qPCR. The correlation between the high proliferation category of breast cancers having a Ki-67 LI of ≥14 per cent as suggested by Saint Gallen consensus conference of 2011[9] with the RT-qPCR value of Ki-67 mRNA was also found to be significant. Similarly, a significant correlation was seen when comparison was drawn between the high LI group opined by the Saint Gallen consensus conference of 2013[10] and the recommendations of Ohara *et al*[13]. For the purpose of analysis, the recommended cut-offs by Bustreo *et al*[14] were dichotomously grouped into low- and intermediate-risk category (with a Ki-67 LI including values till 19%) and high-risk category (with a Ki-67 LI of ≥20%). The high-risk category showed a significant correlation with the corresponding category of RT-qPCR having a Spearman's correlation coefficient of 0.492 (*P*<0.001). In this study, nine patients were found to have high proliferation (>25%) by IHC, but low by RT-PCR (<22.23%), while 14 were found to have high proliferation (>22.23%) by RT-PCR but low by IHC (<25%).

About 21 per cent patients had TNBC, similar to other studies[25,26]. Cohen's kappa value and ICC for Ki-67 LI on IHC with mRNA detection further highlighted the concordance between them, indicating an important link between mRNA and protein

expression. This was in consonance with previous studies[27,28].

These observations coupled with the current observation that mRNA levels have adequate capability to segregate low- and high-risk groups may justify using RT-qPCR for determining the tumour growth fraction as opposed to Ki-67 LI assessment by IHC which lacks standardization, objectivity and interlaboratory comparability. Marme *et al*[29] concluded that Ki-67 mRNA expression was more objective and highly reproducible and less prone to vagaries of pre-analytical, analytical and post-analytical inconsistencies and was more meaningful than Ki-67 protein expression both on visual and image analysis quantifications. Wirtz *et al*[20] on multivariate analysis found cancer Ki-67 mRNA content to have independent influence on distant disease-free survival (adjusted HR: 0.51, 95% CI: 0.29-0.89, *P*=0.019) while Ki-67 protein expression had no influence (*P*=0.266). Sinn *et al*[15] also concluded that RT-qPCR was a more sensitive and specific platform compared to IHC for demonstrating Ki-67 expression in breast cancers. In accordance with these studies, Prihantono *et al*[30] opined that Ki-67 expression detected by both IHC and RT-qPCR was a predictor of clinical response to neoadjuvant chemotherapy. Ács *et al*[31] concluded that Ki-67 expression levels could not be used as the sole parameter in distinguishing non-responsive cases from cases achieving complete pathological response to therapy, as a significant proportion of cases falling in the high Ki-67 group did not achieve complete response, in opposition to what was expected. However, the significance of Ki-67 as a prognostic and predictive marker was unquestionable.

The optimal cut-off obtained by ROC curve analysis for RT-qPCR in this study was 22.23 per cent. This cut-off can effectively be employed to allow for a clear separation of breast cancers into high and low proliferation groups. While this study was limited by small sample size and the relationship of Ki-67 mRNA levels with survival or response to chemotherapy was not studied, this binary separation may be a valuable tool to prognosticate as alluded earlier[29,30].

In conclusion, the study demonstrated a significant correlation between Ki-67 LI determined by IHC and mRNA determination by RT-qPCR. While IHC requires stringent control of all processes and extensive training of reporting personnel to reduce subjectivity, the quantification of Ki-67 mRNA demands less oversight and precludes human assessment and may be superior in predicting distant disease-free survival compared to Ki-67 protein expression.

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***Conflicts of Interest*:** None.

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