Article published online: 2020-12-14

Droplet digital polymerase chain reaction offers an improvisation over conventional immunohistochemistry and fluorescent *in situ* hybridization for ascertaining Her2 status of breast cancer

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Abstract

Background: Droplet digital polymerase chain reaction (DDPCR) is a recent modality for detecting Her2 expression which is quantitative, cheaper, easier to standardize, and free from interobserver variation. **Purpose:** The purpose of this study is to incorporate DDPCR in the current diagnostic paradigm with clinical benefit. **Materials and Methods:** Fifty-four consecutive patients were tested by immunohistochemistry (IHC), fluorescent *in situ* hybridization (FISH), and DDPCR. With FISH result as gold standard, receiver operating characteristic curves for DDPCR ratio were analyzed to label Her2-negative, equivocal, and positive cases as DDPCR score 1, 2, and 3, respectively. Proportion of patients labeled unequivocally as Her2 positive or negative was defined to have "clinically benefitted" from the test. Drawing parallel to inter-relationships between DDPCR, IHC, and FISH in the test cohort, four diagnostic pathways were defined – (1) initial IHC followed by FISH, (2) initial DDPCR followed by FISH, (3) initial IHC followed by DDPCR followed by FISH, and (4) initial DDPCR followed by IHC followed by FISH. **Results:** Clinical benefit of DDPCR as an initial test in the test cohort was 57%, while it was 65% if used as a second-line test among those with an initial inconclusive IHC result. Sensitivity analysis in the simulation cohort revealed that if DDPCR cost was ≤0.6 times the cost of IHC, then a three-step pathway with DDPCR upfront would near certainly prove most cost beneficial. If DDPCR cost was >0.6 but ≤2 times the cost of IHC, then a three-step pathway with DDPCR as second-line test had a higher probability to prove most cost beneficial. If DDPCR cost was >2 times the cost of IHC, then conventional pathway had a higher probability to prove most cost-effective. **Conclusion:** Incorporating DDPCR in the current clinical diagnostic paradigm has the potential to improve its cost-effectiveness and benefit.

Key words: Breast cancer, droplet digital polymerase chain reaction, fluorescent *in situ* hybridization, formalin fixed paraffin embedded, Her2, immunohistochemistry, RNA

Introduction

Breast cancer is the most common cancer among women with 1.6 million new cases and approximately half million deaths per year.[1] One-fifth of these cancers are Her2-positive which follows a more aggressive course.[2] Over the last 15 years, we have made significant progress in treating these patients with Her2-targeted therapy.[3] Her2 assessment can be done at DNA, RNA, and protein levels. The College of American Pathologists/American Society of Clinical Oncology (CAP/ASCO) recommends immunohistochemistry (IHC) to detect Her2 positivity at protein level, combined, in equivocal cases by *in situ* hybridization (ISH) assay with chromogenic or fluorescent probes (FISH, gold standard conventional pathway) for assessing Her2 at DNA level.[2,4] However, both tests are criticized for being cumbersome, expensive, time-consuming, and difficult to standardize.[5,6] Concerns have also been raised about reproducibility of Her2 protein assessment by IHC in formalin-fixed tissues.[7] In contrast, polymerase chain reaction (PCR)-based methodologies do not have the issue of interobserver variation as they involve quantitative measurements to detect Her2 positivity at DNA and RNA level and thus can be easily standardized and automated.[8-12]

Most of the previous experiences with PCR-based tests utilized real-time PCR (RTPCR), and results of concordance with FISH have been mixed.[13,14] However, an issue with PCR-based testing that needs to be understood is that while FISH categorizes almost all patients as "positive" or "negative," PCR-based tests return a continuous score. Thus, we need to define cutoffs while comparing against a gold standard, which renders many patients being assigned an "equivocal" category. However, to the stakeholders, all that matter are a clear-cut "positive" or "negative" diagnosis at the end of a diagnostic pathway, which we define as "clinical benefit," as an "equivocal" score forces them to opt for a second-line test. None of the conventional statistical indices such as "concordance" and "percentage positive or negative agreement" are able to uniquely capture this phenomenon effectively.

Droplet digital PCR (DDPCR) is a recent modality of detecting genetic abnormality at DNA and RNA levels.[15,16] Majority of the previous studies on DDPCR have utilized genomic DNA and encouraging "concordance" with conventional tests have been reported.[17,18] However, as explained, "concordance" can be artificially manipulated to any value by choosing arbitrary cutoffs of continuous scores offered by PCR-based tests. Any research should ultimately translate to clinical and cost benefit for the stakeholders, and none of the previous studies have explored the "clinical benefit" of DDPCR in the prevalent clinical paradigm of diagnosing breast cancer patients. We were the first to recently assess utility of RNA expression in assessing Her2 status using RTPCR in terms of "clinical" and "cost benefit" to the patient.[19] RTPCR's performance was compared against IHC, both as a first-line test, and among those with IHC score 2 as a reflex second-line test. Results were disappointing as "clinical benefit" of RTPCR as a first- and second-line test was only 15.7% and 14%, respectively. However, DDPCR is more sensitive, has lesser

Access this article online
Quick Response Code:
Website: www.sajc.org
DOI: 10.4103/sajc.sajc_344_18

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How to cite this article: Suryavanshi M, Jaipuria J, Mehta A, Kumar D, Panigrahi MK, Verma H, *et al*. Droplet digital polymerase chain reaction offers an improvisation over conventional immunohistochemistry and fluorescent in situ hybridization for ascertaining Her2 status of breast cancer. South Asian J Cancer 2019;8:203-10.

quantitative variability, and does not require calibration curves before actual samples are run as opposed to RTPCR.[20,21] This is because DDPCR is directly dependent upon both the number of absolute replicate measurements and the template concentration while RTPCR needs known calibrators. Thus, using a protocol similar to our previous study, we aimed to assess if DDPCR could offer a viable alternative for improvisation over conventional IHC and FISH testing for ascertaining Her2 status of breast cancer.

Materials and Methods

Ours is a tertiary care regional cancer center based in northern India. Annually more than 300 primary unilateral treatment-naive breast cancer cases are treated by our medical oncology service.[22] Our conventional protocol for breast cancer diagnosis and Her2 expression testing is described elsewhere and is in agreement with 2013 CAP/ASCO guidelines.[19] Since this study was aimed to ascertain characteristics of DDPCR as a diagnostic test, after the Institutional Ethics Committee approval, we decided to perform IHC, FISH, and DDPCR on all consecutive primary treatment-naive cases for an arbitrary duration of 2 months from June to August 2017.

Tissue processing protocol

The immunostaining procedures were performed using formalin-fixed paraffin-embedded (FFPE) tissue sections. RTU antibodies were used for Her2 (rabbit monoclonal antibody 4B5) on Ventana, Arizona, USA, automated IHC slide stainer BenchMark XT. FISH reaction was performed with PathVysion Her2 DNA Probe Kit (Abbott Molecular, Illinois, USA) according to the manufacturer's instructions. Analysis of Her2/CEP17 signals was done using Leica DM 6000B, Germany, and results were scored as "positive" (Her2:CEP-17 ratio >2 with an average Her2 copy number >4 signals/cell or Her2:CEP 17 >2 with an average Her2 copy number <4 signals/cell or Her2:CEP17 ratio <2 with an average Her2 copy number >6 signals/cell), "negative" (Her2:CEP17 ratio <2 an average Her2 copy number <4 signals/cell), or "equivocal" for Her2 amplification (Her: CEP17 ratio <2 with an average Her2 copy number >4 and <6 signal/cell).

For RNA assessment, FFPE tissues from breast tumor samples with more than 30% tumor tissue on visual examination were obtained. RNA was isolated using Promega ReliaPrep FFPE Total RNA Miniprep System (Wisconsin, USA). cDNA was generated using Omniscript RT-PCR Kit (Qiagen, Germany). Her2 mRNA expression levels from total RNA were determined using TaqMan hydrolysis probe on Bio-Rad QX200 DDPCR platform, California, USA. β-actin was taken as control gene [Table 1].

The DDPCR workflow was done by partitioning the TaqMan (Applied Biosystems, Massachusetts, USA) reaction mix containing sample cDNA into aqueous droplets in oil through the QX100 Droplet Generator. After transfer of droplets to a 96-well PCR plate, a two-step thermocycling protocol followed (95°C for 10 min; 40 cycles of (94°C for 30 s, 55°C for 60 s)) and final step at 98°C for 10 min was carried out in a conventional thermal cycle. Bio-Rad T100; and the PCR plate was then transferred to the QX200 Droplet Reader (a droplet flow cytometer) for automatic reading of samples in all wells. Bio-Rad QX200 reagents and consumables were used for the experiments including droplet generator oil (186–3005), DG8 cartridges (186–4008) and gaskets (186–3009), droplet reader oil (186–3004), and DDPCR supermix for probes (186–3010). About 10 ng of cDNA (3–-4 ng/μl concentration) was taken for amplification. Final concentrations of primer probes used for DDPCR were taken as 200 nM each primer, 100 nM each probe in a 20-μl reaction. β-actin was taken as control gene for normalization [Table 1]. The values of Her2 expression were calculated using absolute Her2 (copies/μl)/β-actin (copies/μl) and referred as "DDPCR ratio."

Clinical utility assessment

As already described, "clinical benefit" of a test was defined as proportion of patients labeled conclusively as Her2 positive or negative, as an equivocal result forces the patient to opt for a second-line test. Clinical utility assessment was done in three steps, details of which can be found elsewhere;[19] however, a brief summary is provided below.

First step – determining criteria to define droplet digital polymerase chain reaction categories in the test cohort

Receiver operating characteristic (ROC) curves for DDPCR ratio with FISH outcome as classification variable were analyzed, and subject to values satisfying the criteria corresponding to ≤100 sensitivity and >100% specificity, Her2- "negative," "equivocal," and "positive" patients were assigned a DDPCR score of 1, 2, and 3, respectively. Thus, 1 and 3 comprised a "conclusive DDPCR result category" and "clinically benefitted" from testing.

Second step – simulating data for 1 year to form simulation cohort and defining diagnostic pathways

Data from 2 months were simulated for 1-year subject to following conditions so as to reflect common experience,[22] 25% overall Her2 positivity,[21] and distribution of IHC categories in accordance with results from Bayesian network meta-analysis of unbiased literature reports by Dendukuri *et al*.[23] Two broad categories of diagnostic pathways with four possibilities were then defined – Two-step pathway comprising (A1) initial IHC followed by FISH for equivocal cases (conventional pathway) and (A2) initial DDPCR followed by FISH for equivocal cases and three-step pathway comprising (B1) initial IHC followed by DDPCR for an equivocal IHC score followed by FISH for an equivocal DDPCR test result and (B2) initial DDPCR followed by IHC for an equivocal DDPCR score followed by FISH for an equivocal IHC test result.

Third step – comparing clinical benefit, incremental cost-effectiveness ratio, and cost benefit for diagnostic pathways in the validation cohort

Clinical benefit of next to last step of two- and three-step pathways was compared. Approach with higher clinical benefit was considered to "dominate" the other. For pathways with initial IHC, incremental cost-effectiveness ratio (ICER) for subsequent FISH or DDPCR followed by FISH was compared from a provider's perspective. Sensitivity analysis was performed to determine probability of three-step pathway incorporating DDPCR as second-line test to remain "cost-effective" over conventional pathway with varying iterations of input parameters. Sensitivity analysis was also performed to compare "cost benefit" of both three-step pathways with varying iterations of input parameters.

Statistical analysis

Quantitative variables were reported as median (interquartile range) and compared using Mann–Whitney U-test, while categorical variables were presented as numbers (proportion). ROC curve analysis was done to assess discriminative ability of DDPCR in identifying Her2-positive cases and define "score categories" as explained previously. Area under the ROC curve (AUC) was reported along with 95% binomial exact confidence interval (CI) and standard error (SE) (MedCalc Statistical Software version 15.8 [MedCalc Software bvba, Ostend, Belgium]). Dendukuri *et al.*[23] in their meta-analysis determined median 36.1%, 35.5%, 12.2%, and 16.2% patients to be distributed in IHC 0, 1, 2, and 3 categories, respectively. Constraint to overall 25% Her2 positivity and 100% concordance with FISH for "conclusive" IHC categories (categories 0, 1, and 3), straightforward arithmetic logic was used to simulate population for 1 year (assuming 320 patients). For cost-effectiveness analysis, ICER between conventional and three-step pathways was calculated using the following formula:

$$ICER = (Cost_{new} - Cost_{old})/(Effectiveness_{new} - Effectiveness_{old})$$

Foe sensitivity analysis to account for variability in the real world, arithmetic logic was used to derive general equations for ICER and cost benefit (calculated by determining cost difference). Monte Carlo simulation with 100,000 repetitions was used to obtain cumulative distribution plots (IBM SPSS Statistics for Windows v 25.0 [IBM Corp., Armonk, NY, USA]). All costs were calculated relative to cost of one IHC test assumed to be one unit. Cost of one FISH test at our hospital is 3 times the cost of one IHC test, and the same has been reported by others.[23,24] Two-tailed alpha <0.05 was set as statistically significant beforehand.

Results

Fifty-four patients comprised the study test population, and median age was 52 years. IHC, FISH, and DDPCR were performed for all patients. While none with IHC score of 0 or 1 was FISH positive for Her2, all of the 15 cases with IHC score of 3 were FISH positive. Twenty-six out of 54 cases were assigned IHC score of 2. Thus, 28 (54 − 26) patients benefitted from initial IHC testing (clinical benefit 52% [28/54]) with overall concordance between IHC and FISH being 100% (28/28) for those with IHC score of 0, 1 and 3 (conclusive IHC categories).

Determining criteria to define droplet digital polymerase chain reaction categories in the test cohort

On ROC curve analysis (Appendix 1 for raw data tables), DDPCR score >0.059 had 100% sensitivity while score >1.018 had 100% specificity. Twenty-three cases were assigned that scores intermediate between 0.059 and 1.018. Thus, 31 (54 – 23 = 31) patients would have benefitted from DDPCR testing if it was used as a first-line test (clinical benefit: 57%) with concordance between DDPCR and FISH being 100% for those with DDPCR score ≤0.059 and >1.018 with no false-negative or false-positive cases. Thus, DDPCR could discriminate Her2-positive cases with AUC of 0.91, 95% CI 0.80–0.97, SE 0.034, $P < 0.0001$. Table 2 describes the clinical details including IHC, FISH, and DDPCR distribution of the entire study population. Median DDPCR ratio was significantly lower for Her2-negative cases. If DDPCR would have been used as a second-line test among those with IHC score 2 ($n = 26$), then only 9 patients would have been assigned an inconclusive category translating to a clinical benefit of 65% ([26 – 9]/26) as a second-line test.

Table 1: Primer probes for Her2 and B actin

Primers	Sequence
Her2 FP	5'-CCA GGA CCT GCT GAA CTG GT-3'
Her2 RP	5'-TGT ACG AGC CGC ACA TCC-3'
Her2 Probe	FAM-5'-CAG ATT GCC AAG GGG ATG AGC TAC CTG-3'-TAMRA
B-ACTIN FP	5'-CCA CAC TGT GCC CAT CTA CG-3'
B-ACTIN RP	5'-AGG ATC TTC ATG AGG TAG TCA GTC AG-3'
B-ACTIN Probe	FAM-5'-ATG CCC TCC CCC ATG CCA TCC TG-3'-TAMRA

Table 2: Demographic and clinical details of entire study population (n=54)

Variable	Result	P
Age, years, median (IQR)	52 (46-58)	
Her2/neu positivity by FISH, *n* (%)	30 (56)	
DDPCR performance		
DDPCR ratio in Her2/neu-positive patients*, median (IQR), n=30	1.90 (0.64-7.02)	***<0.0001***
DDPCR ratio in Her2/neu-negative patients*, median (IQR), n=24	0.066 (0.039-0.30)	

IHC versus DDPCR versus FISH distribution (*n*)

IHC category	DDPCR category			FISH category
	1	2	3	
0 (n=7)	5	2	0	**0**
1 (n=6)	3	3	0	**0**
2 (n=26)	4	7	0	**0**
	0	2	13	**1**
3 (n=15)	0	9	6	**1**

*Assuming FISH as gold standard. FISH category defined as 0=Her 2 negative, 1=Her 2 positive, Significant *P* values marked bold and italicized. FISH=Fluorescent *in situ* hybridization, IQR=Interquartile range, DDPCR=Droplet digital polymerase chain reaction, IHC=Immunohistochemistry

Simulating data for 1 year and determining clinical benefit for diagnostic pathways in the simulation cohort

Table 3 describes the simulated cohort for 1 year (n = 320) and the arithmetic logic behind it. Overall, clinical benefit of IHC as a first-line test was 87.8% (281/320) while that of DDPCR as a first-line test was 59% (189/320). If DDPCR was to be used as a second-line test among those with inconclusive IHC score (n = 39), then its clinical benefit was 74% (29/39) with 10 patients assigned an inconclusive DDPCR score (DDPCR category = 2). If IHC was used as a second-line test among those with inconclusive DDPCR score (n = 131), then its clinical benefit was 92.4% (121/131) with 10 patients assigned an inconclusive IHC score (IHC category = 2). Thus, overall clinical benefit of IHC followed by DDPCR as well as DDPCR followed by IHC was the same (96.9% [310/320]), and two-step pathways were dominated by three step pathways.

Deriving equations comparing incremental cost-effectiveness ratio and cost benefit for diagnostic pathways in the simulation cohort and sensitivity analysis

Arithmetic logic and equations for ICER and cost-benefit analysis comparing various pathways are described in Table 4. For sensitivity analysis, Monte Carlo simulation was performed

Table 3: Immunohistochemistry, fluorescent *in situ* hybridization, and droplet digital polymerase chain reaction category distribution of patients in the simulated validation cohort (*n*=320)

IHC category	DDPCR category			FISH category
	1	2	3	
0 (*n*=115)	82	33	0	0
1 (*n*=114)	57	57	0	0
2 (*n*=39)	4	7	0	0
	0	3	25	1
3 (*n*=52)	0	31	21	1

Simulated population cohort was constructed subject to following constraints: 1. Total population size=320, 2. Overall Her2 positivity=25% (meaning 80 patients), 3. Distribution of IHC 0, 1, 2, and 3 categories to be 36.1%, 35.5%, 12.2%, and 16.2%, respectively, as determined by Dendukuri *et al*. (meaning 115, 114, 39, and 52 patients, respectively), 4. Concordance of 100% between FISH and IHC for IHC categories 0, 1, and 3 (meaning all 52 patients of IHC category 3 to be FISH-positive patients and rest 80-52=28 FISH-positive patients be assigned to IHC category 2), 5. DDPCR category distribution in conclusive IHC categories (0, 1, and 3) to be in proportion to those observed in test cohort (meaning if 7 patients in IHC 0 category in the test cohort were distributed in the ratio 5:2:0, then corresponding patients in the simulated cohort with 115 patients in IHC 0 category be distributed similarly), and 6. DDPCR category distribution in inconclusive IHC category 2 be in accordance with FISH distribution in IHC category 2 in the test cohort (meaning if 15 FISH-positive patients in IHC 2 category in the test cohort were distributed in ratio 0:2:13, then distribution among 28 FISH-positive IHC category 2 patients in validation cohort be correspondingly similar). DDPCR=Droplet Digital polymerase chain reaction, IHC=Immunohistochemistry, FISH=Fluorescent *in situ* hybridization

with the following constraints (1) cost of FISH normally distributed with mean 3 and standard deviation 0.25, (2) cost of IHC fixed at 1, (3) clinical benefit of DDPCR as a first-line test uniformly distributed in the interval 0.54–0.65, (4) clinical benefit of DDPCR as a second-line test uniformly distributed in the interval 0.58–0.87, (5) clinical benefit of IHC as a first-line test uniformly distributed in the interval 0.84–0.91, (6) clinical benefit of IHC as a second-line test uniformly distributed in the interval 0.86–0.96, (7) fixed DPPCR cost with iterations 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 2.25 for ICER difference, and (8) fixed DDPCR cost with iterations 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 for cost-benefit analysis.

Cumulative distribution plot in Figure 1 showed that a three-step pathway with DDPCR as a second-line test had 68% probability to prove more cost-effective than conventional pathway if the cost of DDPCR was 2 times the cost of IHC. Cumulative distribution plot in Figure 2 showed that a three-step pathway with DDPCR upfront could have higher cost benefit than a three-step pathway with immunohistochemistry upfront provided the cost of DDPCR was <0.5 times the cost of immunohistochemistry. Combined interpretation of the two distribution plots implied that if DDPCR cost was ≤0.6 times the cost of IHC, then a three-step pathway with DDPCR upfront would near certainly prove most cost beneficial. If DDPCR cost was >0.6 but ≤2 times the cost of IHC, then a three-step pathway with DDPCR as second-line test had a higher probability to prove most cost beneficial. If DDPCR cost was >2 times the cost of IHC, then conventional pathway had a higher probability to prove most cost-effective.

Discussion

We tried to evaluate clinical utility of an ultra-sensitive PCR (DDPCR) against IHC both as first-line test and among those with IHC score 2 as a reflex second-line test. We chose to study RNA as it has been shown on a cell line study of breast cancer that different methods of

Table 4: Cost benefit and effectiveness calculations in the validation cohort with explanations

Cost of one IHC test "i"=1 unit
Cost of one FISH test ="f"
Cost of one DDPCR ="d"
Clinical benefit of IHC as an initial test="c_i"=0.878, 95% binomial exact CI*–0.84-0.91
Clinical benefit of DDPCR as an initial test ="c_{1d}"=0.59, 95% binomial exact CI*–0.54-0.65
Clinical benefit of IHC as a second-line test among those with an initial equivocal DDPCR score="c_{i2}"=0.92, 95% binomial exact CI*–0.86-0.96
Clinical benefit of DDPCR as a second-line test among those with an initial equivocal IHC score="c_{2d}"=0.74, 95% binomial exact CI*–0.58-0.87
Total number of patients in study population="x"=320
Clinical benefit of IHC followed FISH for inconclusive IHC=clinical benefit of IHC followed by DDPCR for inconclusive IHC followed by FISH for inconclusive DDPCR=1 (as everyone gets a definitive diagnosis at the end of both pathways)
Cost of initial IHC for entire population=ix
Cost of IHC followed by FISH for inconclusive IHC=$ix + (1-c_i)xf$
Cost of IHC followed by DDPCR for inconclusive IHC followed by FISH for inconclusive DDPCR=$ix + (1-c_i)xd + (1-c_{2d})(1-c_i)xf$
Cost of DDPCR followed by IHC for inconclusive DDPCR followed by FISH for inconclusive IHC=$dx + (1-c_{1d})xi + (1-c_{i2})(1-c_{1d})fx$
$ICER_{\text{IHC versus IHC followed by FISH}}=(ix + [1-c_i]xf - ix)/(1-c_i)=xf$
$ICER_{\text{IHC versus IHC followed by DDPCR followed by FISH}}=(ix + [1-c_i]xd + [1-c_{2d}][1-c_i]xf - ix)/(1-c_i)$
$=xd + (1-c_{2d})$
$xf=xf+xd-xfc_{2d}$
For a three-step pathway with DDPCR as second-line test to have superior "cost-effectiveness" over conventional pathway
$ICER_{\text{IHC versus IHC followed by DDPCR followed by FISH}}<ICER_{\text{IHC versus IHC followed by FISH}}\Rightarrow xf+xd - xfc_{2d}< xf$
$\Rightarrow xd< xfc_{2d}$
$\Rightarrow d < fc_{2d}$
$\Rightarrow fc_{2d}-d >0$
For a three-step pathway with DDPCR upfront to have superior "cost benefit" over a three-step pathway with IHC upfront – Cost of IHC followed by DDPCR for inconclusive IHC followed by FISH for inconclusive DDPCR > Cost of DDPCR followed by IHC for inconclusive DDPCR followed by FISH for inconclusive IHC
$\Rightarrow ix + (1-c_i)xd + (1-c_{2d})(1-c_i)xf > dx + (1-c_{1d})xi + (1-c_{i2})(1-c_{1d})fx$
$\Rightarrow ix + (1-c_i)xd + (1-c_{2d})(1-c_i)xf - dx - (1-c_{1d})xi - (1-c_{i2})(1-c_{1d})fx >0$

*Calculated from - http://www.sample-size.net/confidence-interval-proportion. DDPCR=Droplet digital polymerase chain reaction, IHC=Immunohistochemistry, FISH=Fluorescent *in situ* hybridization

next-generation sequencing (NGS) demonstrate different Her2 copy numbers at genomic DNA level, theoretically implying unreliability of DNA based testing in borderline cases.[22] Testing Her2 translation at protein level can also be theoretically problematic as uncommonly, abnormal proteins are degraded *in vivo*. Further, there may exist patients whose Her2 is not amplified and yet is overexpressed by other mechanisms. Previous data on RNA expression of Her2 are predominantly available for RTPCR, and comparison with different analytical methods such as IHC and FISH has been conflicting. Some authors have reported inadequate concordance and increased number of false negatives,[13,25-30] while others reported good concordance.[31,32] As mentioned earlier, concordance is something that can be artificially manipulated by choosing a particular cutoff, and true value

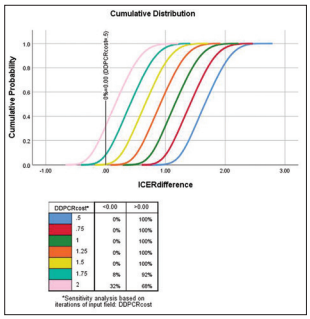

Figure 1: Cumulative distribution plot of incremental cost-effectiveness difference with varying fixed costs of droplet digital polymerase chain reaction. Three-step pathway with droplet digital polymerase chain reaction as a second-line test would be more cost-effective than conventional pathway if cost of droplet digital polymerase chain reaction was <1.5 times the cost of immunohistochemistry. For higher costs, the probability lowered but remained encouraging. When droplet digital polymerase chain reaction cost was 1.25 times the cost of immunohistochemistry, then the contribution of "cost of fluorescent *in situ* hybridization" and "clinical benefit of droplet digital polymerase chain reaction as a second-line test" to the overall variance was 66.19% and 36.29%, respectively. ICER=Incremental cost-effectiveness ratio, DDPCR=Droplet digital polymerase chain reaction

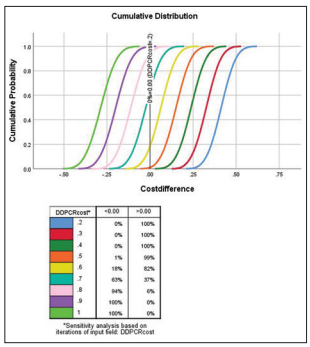

Figure 2: Cumulative distribution plot of cost benefit comparing a three-step pathway with droplet digital polymerase chain reaction upfront versus a three-step pathway with immunohistochemistry upfront, with varying fixed costs of droplet digital polymerase chain reaction. A three-step pathway with DDPCR upfront could have higher cost benefit than a three-step pathway with immunohistochemistry upfront provided the cost of DDPCR was <0.5 times the cost of immunohistochemistry. When droplet digital polymerase chain reaction cost was fixed at 0.5 times the cost of immunohistochemistry, then the contribution of "clinical benefit of droplet digital polymerase chain reaction as a first-line test," "clinical benefit of droplet digital polymerase chain reaction as a second-line test," "clinical benefit of immunohistochemistry as a first-line test," "clinical benefit of immunohistochemistry as a second-line test", and "cost of fluorescent *in situ* hybridization" to the overall variance was 36%, 20.7%, 15.7%, 27.7%, and 0.47%, respectively. DDPCR=Droplet digital polymerase chain reaction

of a test is only known by assessing clinical benefit to the patient. Further, certain studies have questioned the use of RTPCR values in equivocal and positive cases for clinical use.[33] Microarray,[34] NanoString,[35] and NGS[36] are newer platforms for gene expression profiling; however, they are expensive and cumbersome, precluding their use outside multigene analysis.

DDPCR is an important methodology in translational cancer research because of its superior sensitivity and accuracy for detection of genomic DNA or RNA expression.[37] The advantages of DDPCR over RTPCR have already been described. The pros and cons of DDPCR versus IHC as a test are explained in Table 5.

DDPCR can be done on both fresh frozen and formalin-fixed tissues. Majority data for Her2 detection by DDPCR on FFPE samples are from breast and gastric cancers, showing good correlation among DDPCR, IHC, and FISH testing.[17,38-41]

Wang *et al*. described their experience with FFPE samples for detecting Her2 amplification at DNA level in breast and gastric cancers by DDPCR and showed 94.4% concordance with

Table 5: The pros and cons of droplet digital polymerase chain reaction versus immunohistochemistry as a diagnostic test

Factors	DDPCR	IHC
1. Quantitative measurement	√	×
2. Easy standardization	√	×
3. Interobserver variation	×	√
4. Lesser cost	√	×
5. Commonly used method	×	√

DDPCR=Droplet digital polymerase chain reaction, IHC=Immunohistochemistry

IHC.[42] Another study on 102 invasive breast cancer samples by Wang *et al*. using two digital platforms DDPCR and RainDance for Her2 detection at DNA level (assuming FISH as gold standard) showed a sensitivity and specificity of 82.8% and 97.3% respectively, with a kappa value of 0.833 ($P < 0.001$) in comparison to IHC.[43] On further testing of 114 equivocal IHC cases, 75% (21/28) Her2-amplified and 95% (82/86) Her2 nonamplified cases were correctly classified as positive and negative by DDPCR, respectively.[43]

However, there are very limited data available for detecting Her2 transcript at RNA level, and as explained previously, DNA-based testing for Her2 detection could be unreliable in borderline cases due to differences in detection of Her2 copy numbers by different diagnostic modalities.[18] Furthermore, most of the molecular classifications are based on RNA expression level, and prognostic tests such as Oncotype DX (Genomic Health, USA) and MammaPrint are based on RNA transcript quantification.[44,45] Hence, there is a strong need of more studies assessing Her2 transcript on this robust platform. We could come across only two previous studies assessing Her2 expression at RNA level using DDPCR. Heredia *et al*.[46] assessed Her2 in 12 patients both at DNA and RNA levels with data for FISH reported in only 5 cases, while Meehan *et al*.[18] analyzed 178 cases of breast cancer without reporting FISH data and compared IHC with absolute quantities of mRNA in copies/µl without normalization. Importantly, both studies strongly suggested that mRNA values using DDPCR could be considered as an alternative to IHC. Our study assessed RNA after normalizing with a reference gene and also correlated all cases with FISH findings. In fact, our study went the complete way to assess all cases at DNA, RNA, and protein levels.

Beyond clinical utility, we are the first to evaluate cost-effectiveness of DDPCR in the present diagnostic paradigm for assessing Her2 status in breast cancer. Our results showed that introducing DDPCR as a second-line test in the conventional pathway could improve its cost-effectiveness if the cost of one DDPCR test was ≤2 times the cost of one IHC test. However, in reality, the actual cost of one DDPCR test is likely to be lower than that of one IHC test. In terms of input costs, the quotation of a new DDPCR machine offered to our hospital was 60% of the cost of a new IHC machine. Both IHC and DDPCR require one trained person with 4–6 working h for each run. Further, each run of DDPCR is around 3$ per reaction,[47,48] IHC around 100$–150$, and FISH being double to triple of IHC.[49] Importantly, if cost of one DDPCR test was ≤60% of one IHC test, then a three-step pathway with DDPCR upfront had

higher probability to prove most cost beneficial among all other possible diagnostic pathways.

Conclusion

We performed a comprehensive audit of clinical utility of DDPCR in ascertaining presence or absence of Her2 expression at RNA level in FFPE samples of breast cancer and describe its clinical protocol and cutoffs. Incorporating DDPCR in the current clinical paradigm of assessing Her2 expression in breast cancer has the potential to improve its cost-effectiveness and benefit.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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Appendix

Appendix 1

Receiver operating characteristic curve of droplet digital polymerase chain reaction was analyzed in its ability to discriminate those with Her2 positivity assuming fluorescent *in situ* hybridization as gold standard in the overall population. We chose 100% agreement as cutoff criteria while evaluating performance of droplet digital polymerase chain reaction as immunohistochemistry 0, 1, and 3 had 100% agreement with corresponding fluorescent *in situ* hybridization categories in our study. On receiver operating characteristic curve analysis, patients with droplet digital polymerase chain reaction ratio ≤highest value corresponding to 100% sensitivity were all Her2/neu negative, and those with droplet digital polymerase chain reaction ratio >lowest value corresponding to 100% specificity were all Her2/neu positive. Below is receiver operating characteristic curve analysis classification tables with raw data for droplet digital polymerase chain reaction.

Receiver operating characteristic curve analysis raw data for droplet digital polymerase chain reaction in the test cohort

Variable	DDPCR ratio
	DDPCR ratio
Classification variable	Her2 FISH
Sample size	54
Positive group[a]	30 (55.56%)
Negative group[b]	24 (44.44%)

[a]Her2 FISH=1, [b]Her2 FISH=0, DDPCR=Droplet digital polymerase chain reaction, FISH=Fluorescent *in situ* hybridization

Disease prevalence (%)	Unknown

Area under the receiver operating characteristic curve

AUC	0.922
Standard error[a]	0.0342
95% confidence interval[b]	0.816–0.977
Z statistic	12.330
Significance level *P* (area=0.5)	<0.0001

[a]DeLong method, [b]Binomial exact. AUC=Area under the receiver operating characteristic curve

Youden index

Youden index J	0.7167
Associated criterion	>0.119047619
Sensitivity	96.67
Specificity	75.00

Criterion values and coordinates of the receiver operating characteristic curves

Criterion	Sensitivity	95% CI	Specificity	95% CI	+LR	−LR
≥0.005886525	100.00	88.4-100.0	0.00	0.0-14.2	1.00	
>0.005886525	100.00	88.4-100.0	4.17	0.1-21.1	1.04	0.00
>0.014840989	100.00	88.4-100.0	8.33	1.0-27.0	1.09	0.00
>0.017834395	100.00	88.4-100.0	12.50	2.7-32.4	1.14	0.00
>0.018567639	100.00	88.4-100.0	16.67	4.7-37.4	1.20	0.00
>0.028340081	100.00	88.4-100.0	20.83	7.1-42.2	1.26	0.00
>0.038769231	100.00	88.4-100.0	25.00	9.8-46.7	1.33	0.00
>0.039330544	100.00	88.4-100.0	29.17	12.6-51.1	1.41	0.00
>0.043195266	100.00	88.4-100.0	33.33	15.6-55.3	1.50	0.00
>0.043650794	100.00	88.4-100.0	37.50	18.8-59.4	1.60	0.00
>0.049115914	100.00	88.4-100.0	41.67	22.1-63.4	1.71	0.00
>0.05625	100.00	88.4-100.0	45.83	25.6-67.2	1.85	0.00
>0.059217877	100.00	88.4-100.0	50.00	29.1-70.9	2.00	0.00
>0.070488722	96.67	82.8-99.9	50.00	29.1-70.9	1.93	0.067
>0.072604284	96.67	82.8-99.9	54.17	32.8-74.4	2.11	0.062
>0.106635071	96.67	82.8-99.9	58.33	36.6-77.9	2.32	0.057
>0.109677419	96.67	82.8-99.9	62.50	40.6-81.2	2.58	0.053
>0.11023622	96.67	82.8-99.9	66.67	44.7-84.4	2.90	0.050
>0.112698413	96.67	82.8-99.9	70.83	48.9-87.4	3.31	0.047
>0.119047619	96.67	82.8-99.9	75.00	53.3-90.2	3.87	0.044
>0.177027027	93.33	77.9-99.2	75.00	53.3-90.2	3.73	0.089
>0.368877867	90.00	73.5-97.9	75.00	53.3-90.2	3.60	0.13

Contd....