RESEARCH ARTICLE

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Diagnostic Accuracy of Immunohistochemistry for HER2-Positive Breast Cancer

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Abstract

Objective: Currently, human epidermal growth factor receptor 2 (HER2)-positive breast cancer cells are diagnosed under the American Society of Clinical Oncology (ASCO) and College of American Pathologists (CAP) 2018 guidelines. The guideline combined the results of in situ hybridization (DISH) and immunohistochemistry (IHC) techniques. The IHC technique is easy, cheap, and suitable for developing country. Therefore, in this study, we validated the use of IHC alone compared to the results of HER2 amplification under ASCO/CAP 2018 guidelines in diagnosed HER2 positive breast cancer cells. **Methods:** A total of 510 breast cancer tissue samples from Rajavithi Hospital in Bangkok, Thailand, from January 1st, 2022, to May 31st, 2023, were analyzed by IHC, followed by dual ISH (DISH). We selected 58 samples of IHC equivocal (score 2+) and 98 samples of IHC positive (score 3+) to analyze the diagnostic values by comparing them to the results of HER2 amplification. **Results:** The HER2 IHC score was found to agree with HER2 amplification with a sensitivity of 87.96%, a specificity of 93.75%, a positive predictive value of 96.94%, a negative predictive value of 77.59%, a positive likelihood ratio of 14.07, a negative likelihood ratio of 0.13, and an accuracy of 89.74%. **Conclusion:** The promising outcomes suggest that a positive IHC test result (score 3+) could potentially stand alone for patients with breast cancer undergoing anti-HER2 treatment, even without DISH confirmation.

Keywords: Immunohistochemistry- dual stain in situ hybridization- HER2- breast cancer

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Introduction

Breast cancer is the most frequent female malignancy in the world, and also in Thailand. This cancer is the leading cause of death, and a significant economic and social concern. In Thailand in 2020, there will be 22,158 new breast cancer diagnoses and 8,266 deaths (Arnold et al., 2022). Despite the great efficacy of screening and early detection methods such as mammograms and breast self-examination in Thailand, the prevalence of breast cancer has been gradually growing (Lakha et al., 2020). Furthermore, 10% to 30% of all breast cancer cases have HER2 protein overexpression or gene amplification (Iqbal and Iqbal, 2014).

The human epidermal growth factor receptor (HER2), also known as HER2/neu, is one of the epidermal growth factor receptors (ErbB) tyrosine kinase receptors (Type I tyrosine kinase receptors). This gene is situated at 17q12 on chromosome 17 (Krishnamurti and Silverman, 2014). HER2 is an oncogene that has a role in cell proliferation and differentiation (Iqbal and Iqbal, 2014). It was involved in the pathogenesis of breast cancer (Ishikawa et al., 2014). HER2 amplification and/or overexpression in breast cancer patients related to aggressive behavior in breast cancer patients, including, poor prognosis, a short disease-free period, and a short survival period (Burstein, 2005; Wang et al., 2015; Cong et al., 2020).

In Thailand, the HER2 status of all breast cancer cases will be evaluated before receiving therapy. The evaluation of HER2/neu involves employing two distinct methods: immunohistochemistry (IHC) to detect protein expression, and fluorescence in situ hybridization (FISH) or dual in situ hybridization (DISH) to measure gene amplification (Gordian-Arroyo et al., 2019). The IHC scored membrane HER2 level as 1+, 2+ and 3+ whereas the ISH measured HER2 amplification as positive and negative. Both approaches followed the 2018 recommendations of the American Society of Clinical Oncologists and College of American Pathologists (ASCO/CAP) (Gordian-Arroyo et al., 2019). In cases of HER2 amplification positivity, the National Health Society of Thailand (NHSO) recommends a targeted therapy regimen including anti-HER2 family medications such as Pertuzumab and Trastuzumab (Lewis Phillips et al., 2008; Gianni et al., 2011; Higgins and Baselga, 2011; Den Hollander et al., 2013; Doval et al., 2021).

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In practice, all patients must first be screened for IHC. In the case of IHC 2+ or 3+, DISH will be done. Since IHC is cheap, quicker, and simpler, ISH is twenty times more complicated, time-consuming, and costly. In situations where there is a lack of ISH confirmation, as often found in developing countries, the results can be enhanced by exclusively depending on IHC. Consequently, this study aimed to determine the concordance rates between IHC scores 2+ and 3+ and HER2 gene amplification. The findings revealed that IHC techniques with a score of 3+ demonstrate comparable results to HER2 amplification, suggesting their potential utility alone without an ISH result.

Materials and Methods

Sample Recruitment

The research utilized formalin-fixed paraffin-embedded (FFPE) tissue blocks obtained from breast cancer tumor cases that had undergone both HER2 IHC and HER2 DISH procedures. These FFPE samples were derived from biopsy specimens taken during the preoperative treatment stage of patients diagnosed with breast cancer. The patients included in the study had primary tumors and had not undergone any previous radiation therapy or chemotherapy. Exclusion criteria were applied for cases with low amounts of pathologic tissue and a lack of clinical data. The diagnosis of invasive ductal carcinoma, histological subtype, estrogen receptor (ER), and progesterone receptor (PR) status were confirmed by KS and SC. Figure 1A and 1D display examples of hematoxylin and eosin (H&E) staining. Clinical data were obtained from the patients' clinical chart records, and all the relevant clinical and histological information is presented in Table 2.

A total of 510 breast cancer cases were initially recruited from the Department of Pathology at Rajavithi Hospital in Bangkok, Thailand, between January 1st, 2022, and May 31st, 2023. After careful selection, 156 breast cancer tissue samples were included for analysis. This hospital-based study protocol was approved by the Institutional Review Board of Rajavithi in Bangkok, Thailand (IRB no. 009/2566), and written informed consent was obtained from all participating patients.

IHC

For IHC, the HER2/neu primary antibodies (4B5) were used. The FFPE blocks were cut into sections with a thickness of 3 µm. The slides were then stained with the HER2/neu (4B5) primary monoclonal antibody (6 µg/100 µl, Ventana Medical Systems, catalog number 790-2991) using an automated slide strainer, the BenchMark Ultra (Ventana Medical Systems, Inc., Arizona, United States). The staining process was conducted at 37°C for 16 minutes. The detection of the HER2 protein was performed using the Ultraview Universal DAB Detection Kit (Ventana-Roche Diagnostics, Meylan, France). Subsequently, the slides were counterstained with Hematoxylin II® (ab245880, Abcam, United Kingdom) for 8 minutes and Bluing Reagent® for 4 minutes (BR-OT, Biogenost, Croatia, EU).

To ensure the accuracy and validity of the staining procedure, positive controls consisting of breast tissue samples known to be HER2-positive were included in each examination. The staining scores were determined by evaluating membrane staining in tumor cells. Based on the 2018 ASCO/CAP criteria, the IHC scores were classified as negative (score of 0 or 1+), equivocal (score of 2+), or positive (score of 3+) (Gordian-Arroyo et al., 2019). KS and SC conducted blind evaluations and provided scores. Figure 1B illustrates an example of an IHC score of 3+, while Figure 1E demonstrates an example of an IHC score of 2+.

DISH

The FFPE blocks were cut into sections with a thickness of 3 mm. The HER2 gene amplification was determined using the inform HER2 DISH DNA probed cocktail assay (catalog number 800-6043) on the automated VENTANA BenchMark ULTRA platform (Ventana Medical Systems Inc., Tucson, AZ, USA). The procedure involved several steps, including deparaffinization, tissue adjustment, proteinase treatment, and DNA denaturation by heating at 80°C for 8 minutes. Subsequently, the slides were incubated with the VENTANA Silver ISH DNP Detection Kit for HER2 copies (black color) for 48 minutes, followed by the VENTANA Red ISH DIG Detection Kit for chromosome 17 (red color) for 56 minutes. Finally, the slides were counterstained with Hematoxylin II® (ab245880, Abcam, United Kingdom) for 8 minutes and Bluing Reagent® (BR-OT, Biogenost, Croatia, EU) for 8 minutes to enhance visibility and provide contrast.

The DISH analysis was conducted by ST and KS under a microscope. In Figure 1C (DISH positive) and 1F (DISH negative), the red signal represents the probe targeting the chromosome 17 centromere (CEP17), serving as an internal control. The black signal corresponds to the HER2 probe on chromosome 17. The results were evaluated based on the ratio of HER2 signals to CEP17 signals and the average HER2 copy number in the cancer cells, following the criteria set by ASCO/CAP (Gordian-Arroyo et al., 2019).

HER2 gene amplification was classified as "positive" if the HER2/CEP17 signal count ratio was 2.0 or greater, or if the ratio was less than 2.0 but the average number of HER2 signals per cell was 6.0 or higher. A score of "equivocal" was assigned if the HER2/CEP17 signal count ratio was less than 2.0, and the average number of HER2 signals per cell ranged from 4.0 to less than 6.0. A score of "negative" was given if the HER2/CEP17 signal count ratio was less than 2.0, and the average number of HER2 signals per cell was less than 4.0 (Nishimura et al., 2016). KS and SC carried out blind evaluations and provided scores.

Statistics Analyses

The statistical analysis was conducted using version 22.0 of the SPSS software (IBM Corp., Armonk, N.Y., USA). The evaluation of the diagnostic test for IHC positivity (score of 3+) was performed using HER2 amplification as a gold standard, following the 2018 ASCO/CAP guidelines. With a 95% confidence interval,

the following parameters were calculated: sensitivity, specificity, negative predictive value (NPV), positive predictive value (PPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR-) to measure the diagnostic test's accuracy and reliability.

Results

Clinical Characteristics

A total of 510 breast cancer carcinoma tissue samples obtained from Rajavithi Hospital in Bangkok, Thailand, underwent HER2 IHC testing. Among these samples, only those with HER2 IHC scores of 2+ and 3+ were selected to undergo further investigation using DISH. Ultimately, 156 cases met the criteria and were included for further analysis. Out of the 156 cases, 58 samples had an equivocal IHC score of 2+ (indicating equivocal HER2 protein expression), while 98 samples showed a positive IHC score of 3+ (indicating strong HER2 protein expression). These samples were chosen for further investigation, suggesting a focus on cases with significant or uncertain levels of HER2 protein expression for subsequent analysis using DISH.

Table 1 presents a summary of the clinical and pathological findings from the patient cohort. A total of 156 Thai patients diagnosed with breast cancer participated in this study. The patients' median age was 54 years, with an interquartile range (IQR) of 45 to 63 years. Lesions were located on the right side in 45.5% of cases and on the left side in 54.4% of cases. The median tumor size was 3 cm, with an IQR of 1.8 to 4.5 cm. Concerning histological subtypes, the majority (91%) of cases were classified as invasive ductal carcinoma. Histological grading revealed that grade 2 accounted for the largest proportion (37.8%), followed closely by grade 3 (39.7%), and grade 1 (4.49%). In terms of receptor status, the distribution of ER and PR was as follows: ER+PR+ (44.2%), ER-PR- (34%), ER+PR- (16%), and ER-PR+ (5.8%).

HER2 IHC and DISH results

A total of 510 cases of invasive ductal carcinoma were examined by IHC. Among them, 58 cases were classified as HER2 IHC 2+, and 98 cases were categorized as HER2 IHC 3+. This distribution is illustrated in Figure 1B and 1E, representing HER2 IHC equivocal (score 2+) and HER2 IHC positive (score 3+) cases, respectively.

Subsequently, the 156 cases underwent DISH analysis, with the results indicating the average HER2 copy number according to the ASCO/CAP 2018 criteria. Following this analysis, 48 cases were categorized as having positive HER2 amplification, while 108 cases showed negative HER2 amplification. These categories are visualized in Figure 1C and 1F, representing positive and negative HER2 amplification, respectively. To provide a contextual visualization, Figure 1A and 1D present the associated H&E stain images of the cases being discussed.

Diagnostic value of HER2 IHC

We used HER2 amplification from the ASCO/CAP 2018 guidelines as a gold standard. There are 156 cases in total, of which 95 are true positive (HER2 IHC3+/positive HER2 amplification), 45 are true negative (HER2 IHC2+/negative HER2 amplification), three are false positive (HER2 IHC3+/negative HER2 amplification), and thirteen are false negative (HER2 IHC2+/positive HER2 amplification). After calculating the diagnostic test for the HER2 IHC method, Table 2 presents all diagnostic values.

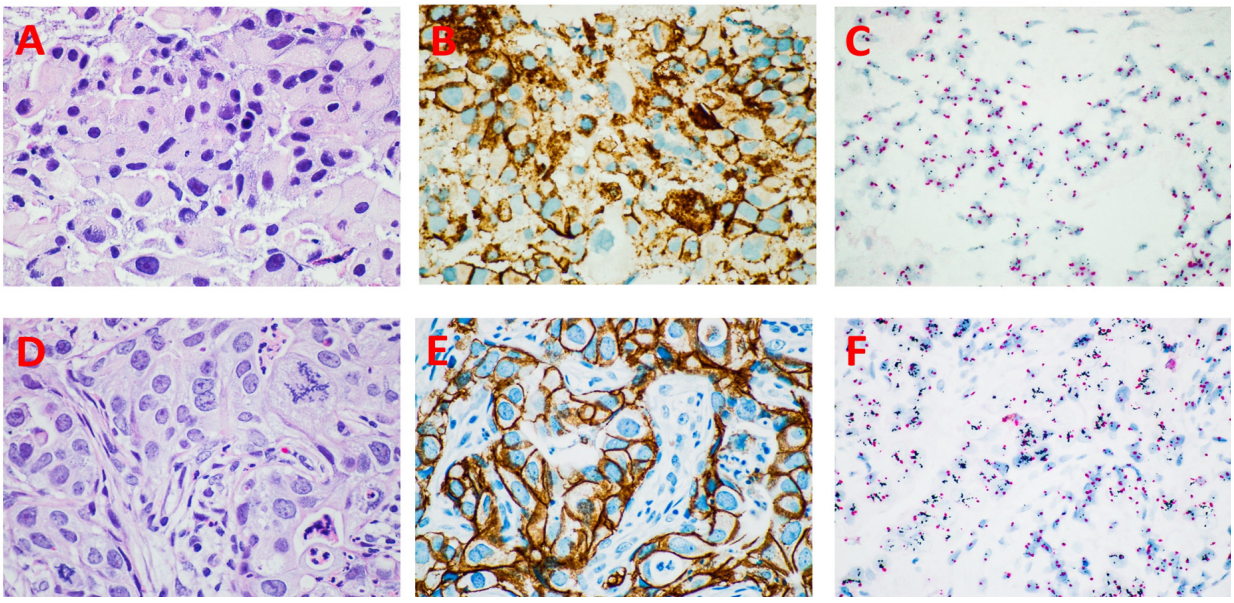

Figure 1. Representative Figures of Results. The upper panel of figures represents the same case with the following images taken at a magnification of 60X: 1A: H&E staining, providing an overview of the tissue sample, 1B: IHC score 3+, indicating a strong HER2 protein expression in the sample, 1C: DISH analysis showing positive HER2 amplification in the sample. On the other hand, the lower panel of figures also depicts the same case, again at a magnification of 60X: 1D: H&E staining, offering a detailed view of the tissue sample, 1E: IHC score 2+, indicating a moderate HER2 protein expression in the sample, 1F: DISH analysis revealing a negative HER2 amplification result in the sample.

Table 1. HER2 IHC Outcome, Diagnostic Sensitivity, and Specificity Percentages

Characteristic	N	HER2 IHC3+	HER2 IHC2+	Positive HER2 amplification	Negative HER2 amplification	Sensitivity (%)	Specificity (%)
Total	156	98	58	108	48	87.96	93.75
Age (year), Median (IQR)	54 (45-63)						
Age group (year), n (%)							
<35	9 (5.8%)	8	1	8	1	100	100
35-50	49 (31.4%)	32	17	36	13	86.11	92.3
51-65	74 (47.4%)	45	29	49	25	87.75	92
>65	24 (15.4%)	13	11	15	9	88.67	100
Tumor laterality, n (%)							
Right	71 (45.5%)	39	32	44	27	84.09	92.59
Left	85 (54.5%)	59	26	64	21	90.63	95.24
Median tumor size (cm) (IQR)	3 (1.8-4.5)						
Tumor size (cm.), n (%)							
< 2	35 (22.43%)	17	18	19	16	84.21	93.75
2-5	53 (33.97%)	29	24	33	20	84.84	95
> 5	19 (12.18%)	16	3	15	4	100	75
No data	49 (31.41%)						
Nodal status, n (%)							
Negative	38 (24.36%)	19	19	21	17	80.95	88.23
Positive	78 (50.00%)	56	22	57	21	96.49	95.23
No data	40 (25.64%)						
Histological subtype, n (%)							
Ductal	142 (91%)	89	53	98	44	87.75	93.18
Lobular	2 (1.3%)	1	1	1	1	100	100
Mammary	3 (1.9%)	2	1	2	1	100	100
Papillary	3 (1.9%)	3	0	3	0	100	100
Other	6 (3.8%)	3	3	4	2	75	100
Histological grade, n (%)							
Grade 1	7 (4.49%)	2	5	2	5	100	100
Grade 2	59 (37.83%)	29	30	34	24	79.41	92
Grade 3	62 (39.74%)	43	19	47	15	89.36	93.33
No data	28 (17.94%)						
Receptor status, n (%)							
ER+, PR+	69 (44.2%)	33	36	39	30	82.05	96.67
ER+, PR-	25 (16%)	13	12	15	10	86.67	100
ER-, PR+	9 (5.8%)	9	0	9	0	100	100
ER-, PR-	53 (34%)	43	10	45	8	91.11	75
HER2 IHC, n (%)							
Equivocal (score 2+)	58 (37.2%)			13	45		
Positive (score 3+)	98 (62.2%)			95	3		
HER2 DISH, n (%)							
Negative	48 (30.8%)	3	45				
Positive	108 (69.2%)	95	13				

The sensitivity, specificity, positive predictive values, negative predictive values, and likelihood ratio positive were very high (87.06%, 93.75%, 77.59%, 96.94%, and 14.07, respectively). In contrast, the likelihood ratio negative was very low (0.13), indicating a strong capability to use HER2 IHC as a screening and diagnostic test. Overall, the accuracy of HER2 IHC in diagnosing HER2 amplification was also high (89.74%), suggesting that we can use the IHC technique as a comparable alternative to DISH to diagnose HER2 amplification.

When examining subgroup criteria such as age, tumor laterality, tumor size, histological subtype, histological

Table 2. Diagnostic Values of HER2 IHC Positive (score 3+) and Equivocal (score 2+)

Diagnostic test	Value	95% CI
Sensitivity	87.96%	80.30% to 93.43%
Specificity	93.75%	82.80% to 98.69%
Positive Likelihood Ratio	14.07	4.69 to 42.19
Negative Likelihood Ratio	0.13	0.08 to 0.21
Positive Predictive Value (*)	96.94%	91.31% to 99.36%
Negative Predictive Value (*)	77.59%	64.73% to 87.49%
Accuracy (*)	89.74%	83.88% to 94.02%

grade, ER, and PR statuses, the IHC HER2 technique demonstrates remarkable effectiveness in terms of sensitivity and specificity, ranging from 75% to 100% across all subgroup analyses (Table 1). Moreover, the IHC HER2 approach has a very high efficiency, especially in cases of metastatic breast cancer, with 96 % sensitivity and 95 % specificity.

Following the likelihood ratio calculation, the HER2 IHC screening was employed to estimate the probability of the individual having the HER2 amplification case. For HER 2 IHC score 3+, the posterior probability of DISH positivity is 97% (with a 95% confidence interval of 91% to 99%). On the other hand, for HER2 IHC score 2+, the posterior probability of DISH positivity is 23% (with a 95% confidence interval of 15% to 32%), as illustrated in Figure 2.

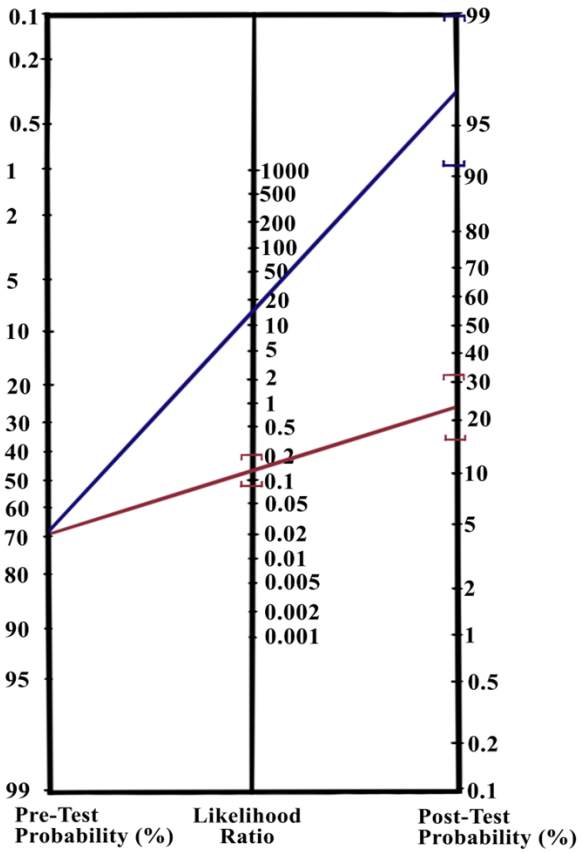

Figure 2. Likelihood Ratio Nomogram Showed the Value of HER2 IHC3+ Test in Diagnosis.

Discussion

IHC assesses the presence of the HER2 protein on tumor cell surfaces. This cost-effective method is readily available in local pathology labs. However, it has certain limitations, including factors influencing results, subjective interpretation, and a notable false positive rate (Pauletti et al., 2000; Tubbs et al., 2001; De Matos et al., 2010). Conversely, FISH or DISH differentiates HER2 copy counts by employing fluorescent-labeled oligonucleotide probes that adhere to precise DNA sections. This genetic methodology yields dependable outcomes with reduced susceptibility to discrepancies among observers. Nonetheless, it constitutes a fee-based analysis and currently entails notable expenses. Furthermore, the turnaround time for results is longer in contrast to IHC. Currently, in Thailand, targeted therapies like Trastuzumab are employed for treating breast cancer patients. To be eligible for such therapies, patients must have an equivocal (score 2+) or positive (score 3+) HER 2 tests through IHC, which should be confirmed by an ISH test like FISH or DISH with a positive outcome.

One limitation of this study is that the IHC scores for HER2 can exhibit variability across different laboratories. This technique can vary due to factors such as the availability of commercial primary antibodies, the time of tissue fixation, and the level of expertise in interpreting HER2 immunostaining (Magaki et al., 2019). For our study, we employed the pathological laboratory at Rajavithi Hospital. The inter-laboratory and intra-laboratory control of the machines and techniques is regularly supervised by the Royal College of Pathologists of Thailand.

Using IHC to detect the HER2 gene yielded a positive result (score 3+) with a specificity of 93.75 %, which is deemed satisfactory. Additionally, it showed high specificity in some clinicopathological subgroups. Previous studies (Gown et al., 2008; Nitta et al., 2008) also demonstrated a strong agreement between IHC and ISH, with an average agreement rate of over 90%. The high specificity of the IHC technique may be due to the revised criteria for reporting and interpreting HER2 IHC by the ASCO/CAP in 2018 (Pasricha et al., 2020). Before 2013, it was not clear what HER2 staining in IHC meant (score 2+). This meant that the tumor cells were slightly to moderately stained, but the cell membrane was not completely stained. But in 2013, the criteria changed to include cancer cells with light to moderately stained areas around the cell membrane. In 2018, the equivocal classification was taken out of in situ hybridization reporting, and only positive and negative classifications were used. Because of these changes, there was less confusing reporting and more accurate reporting ultimately (Gordian-Arroyo et al., 2019; Pasricha et al., 2020).

Anti-Her2 therapies are extensively used and are especially advantageous in situations of breast cancer with negative ER and PR. HER2 status is of utmost clinical significance especially in the case with It serves as a crucial marker for determining whether breast cancer patients should receive trastuzumab, a targeted therapy.

False-negative results in HER2-negative breast cancer patients may lead to the omission of targeted therapy, thereby depriving these patients of potentially beneficial treatment. On the other hand, false-positive results can also pose challenges, as treating too many HER2-negative patients with trastuzumab can result in significant side effects and unnecessary resource waste. Furthermore, anti-HER2 medication is a targeted therapy that is effective in treating HER2-positive metastatic breast cancer. Our findings indicate that the IHC HER2 approach is highly effective in detecting HER2-positive metastatic cases, underscoring the benefits of this technique.

This study demonstrated the agreement between IHC and DISH techniques. The utilization of IHC alone could assist healthcare professionals in the timely and appropriate administration of trastuzumab. This knowledge holds relevance, especially in resource-constrained developing nations. Furthermore, Her2 amplification and/or overexpression have been noted in other malignancies (Menard et al., 2001), implying potential applicability across diverse tumors.

We also found a few incidences of false positives and false negatives from the IHC technique. These can occur at any stage of IHC or tissue fixation, processing, or artifact formation. To combat this, we are attempting to implement more quality controls, such as protocol, antibody, and lab setting. Furthermore, it is crucial to strengthen these findings through the inclusion of larger sample sizes and more diverse cohorts. Expanding our understanding of breast cancer pathogenesis should also involve the incorporation of additional biomarkers such as PIK3CA and P53 mutations, as highlighted in previous studies (Ogeni et al., 2021; Ali et al., 2022). This approach will provide deeper insights into the administration of targeted therapies, leading to improved patient outcomes and a more effective allocation of healthcare resources.

Author Contribution Statement

ST, KS and NK designed the study as well as analyzed, and interpreted data. ST, SC and KS performed the experiments. ST drafted manuscript. NK reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Ethics

This study has been approved by the Institutional Review Board of the Rajavithi Hospital (IRB No. 009/2566). The study has not been conducted as a part of thesis or dissertation.

Availability of data

Data are available by request to the corresponding author.

Data Registry

No prior registration was made for this research.

Statement conflict of Interest

The authors declare that there are no conflicts of interest.

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