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COMPARISON OF IMMUNOHISTOCHEMISTRY (IHC) AND DUAL IN SITU HYBRIDIZATION (DISH) FOR THE ASSESSMENT OF HER2 STATUS IN BREAST CANCER

[1]Tarana Taslima Tithi , [2]Muhammed Alam

[1]Associate professor (Lieutenant Colonel), [2]Associate professor (Lieutenant Colonel)

[1]Department of Histopathology

[1]Armed Forces Institute of Pathology (AFIP), Dhaka, Bangladesh

Abstract

Back ground: HER2 assessment is crucial in guiding therapeutic decisions for breast cancer patients. Dual in situ hybridization (DISH), offering a substitute to fluorescence in situ hybridization (FISH) along with immunohistochemistry, in currently accessible.

Aims: Through dual in situ hybridization (DISH), we can assess and compare the efficacy of Immunohistochemistry (IHC) in determining the HER2/neu expression status of breast cancers.

Methods: This cross-sectional observational research was performed in the Histopathology department of Armed Forces Institute of Pathology (AFIP), Dhaka Cantonment between June 2016 to May 2017 on 55 excised biopsy samples of breast lump diagnosed histopathologically as invasive carcinoma of breast and patients along with metastatic carcinoma of breast to axillary lymph node were included, too. For every case, I verified the histology of the sample examined and identified the areas showing invasive carcinoma. HER2 status was separately tested by IHC and DISH upon two successive 4-mm tissue sections. Statistical analyses of the outcomes was performed by utilizing window build computer software devised along with Statistical Packages for Social Sciences (SPSS-22).

Results: Most of the patients 17(30.9%) belonged to the 41-50 years age group. More than two third 36 (65.5%) patients tumours size was in between 2.1-5.0 cm. The mean HER2/Chr17 ratio was 3.7±3.0 with ranged from 0.7 to 13.1 and the mean average HER 2 number/nucleus was 7.9±5.0 with ranged from 1.2 to 22.6. The validity test of IHC were performed by calculating sensitivity 97.6%, accuracy 76.4%, positive predictive value 76.9% and negative predictive value 66.7% with Dual in Situ Hybridization assumed as the ideal testing method.

Conclusion: DISH emerges as a valuable, straightforward and standardized reproducible technique for identifying HER2/neu gene amplification, particularly in patients along with borderline (2+) immunohistochemistry scores.

***Keywords*:** Immunohistochemistry, Dual in situ hybridization, substitute, ratio, breast cancer.

Introduction:

Human epidermal growth factor receptor 2 (HER2/neu) is a known proto-oncogene, situated at the long arm of human chromosome 17 (17q12), was firstly involved in the development of human breast cancer.[1-2] Researchers stated that various assay techniques have been utilized to identify this over expression, varying from the ancient methods such as Southern or Western Blotting, ELISA to the currently practised immunohistochemistry (IHC), in situ hybridization (ISH) techniques, real-time polymerase chain reaction (PCR) and digital PCR.[3-4] Layfield LJ et al[5] stated that the most commonly utilized techniques for ascertainment of HER2/neu expression status in breast cancers are IHC and fluorescence in situ hybridization (FISH). Both of the methods are linked with technological and interpretive problems. Substitute techniques are present including quantative PCR and the recently developed chromogenic dual in situ hybridization (DISH).[6] Edelweiss M et al[7] assessed the conformity of HER2 status between bight field DISH, FISH, and HER2 IHC accomplished on formalin-fixed CBs (Cell blocks) of recurrent and metastatic breast carcinomas. Stocker A et al[8] concluded that in early breast cancer patients, tumors with high level HER2 amplification ratios (>8), may less possibly react to ideal trastuzumab-containg medicines. This journal aims to provide a comprehensive comparison between IHC and DISH methodologies, shedding light on their respective strengths and limitations in HER2 assessment.

Materials and Methods:

This cross sectional observational study was performed in 55 paraffin tissue blocks and slides along with 1+, 2+ and 3+ IHC score for HER-2 as well as ER and PR slides were collected from the Histopathology department of Armed Forces Institute of Pathology (AFIP), Dhaka Cantonment during study period June 2016 to May 2017. Carcinoma in situ and poorly fixed tissues were precluded from this research. Ethical clearance was acquired from the Research Committee of Armed Forces Institute of Pathology (AFIP). The study's goals, together with its methodology, substitute diagnostic approaches, potential hazards, and advantages, were clarified to the outpatients in a simple and readily comprehensible local language. Subsequently, informed consent was acquired from every participant, and a commitment was made to maintain the confidentiality of all records.

Study Procedure: The specimens were obtained from outpatients along with invasive ductal carcinoma with or without lymph node involvement and undergone surgery. Tumor material comprised of paraffin embedded specimens were fixed in 10% neutral buffered formalin. For every case, I verified the histology of the sample examined and identified the areas showing invasive carcinoma. HER2 status was separately tested by IHC and DISH on two successive 4-mm tissue sections.

Immunohistochemical analysis: Immunohistochemical study was conducted on formalin-fixed and paraffin-embedded tissue sections utilizing the Dako REAL™ Envision™ detection system, adhering to the manufacturer's guidelines. Consultant pathologists accomplished scoring in conformity with the manufacturer company's recommendations.

DISH procedure: Dual in situ hybridization (DISH) was accomplished on individual 4-µm tissue sections for every case using the recently permitted INFORM HER2 Dual ISH DNA Probe Cocktail from Ventana Medical Syatems, following a protocol cleared by the FDA. The staining protocol involved the cohybridization of DNP and DIG-labeled probes to their own specified targets during the DISH procedure. Subsequently, the specimen underwent counterstaining with hematoxylin, facilitating interpretation by light microscopy.

Scoring Criteria:

For IHC: A single observer conducted scoring on all slides, focusing exclusively on the evaluation of membrane staining intensity and pattern utilizing a 0 to 3+ scale, comprising a total of 4 levels: **0** (absence of staining or faint/barely perceptible and incomplete membrane staining within ≤10% of tumor cells), **1** (faint/barely perceptible and incomplete membrane staining within >10% of tumor cells), **2** (incomplete and/or weak/moderate circumferential membrane staining within >10% of tumor cells or intense circumferential staining within ≤10% of tumor cells), and **3** (complete, intense circumferential membrane staining within >10% of tumor cells).

For DISH: The categorization of cases was directed by the 2013 HER2 ASCO/CAP guideline for a dual-color probe wherein if the dual-probe HER2/CEP17 ratio was <2.0 with an average HER2 copy number <4.0 signals/cell, the case was designated as negative for amplification. In cases where the dual-probe HER2/CEP17 ratio was < 2.0 with an average HER2 copy number ≥4.0 and <6.0 signals/cell, the classification was deemed equivocal. If the dual-probe HER2/CEP17 ratio was ≥2.0 with an average HER2 copy number ≥4.0 signals/cell or the dual-probe HER2/CEP17 ratio was ≥2.0 with an average HER2 copy number <4.0 signals/cell, or the dual-probe HER2/CEP17 ratio was <2.0 with an average HER2 copy number ≥6.0 signals/cell, the case was considered to be amplified.

All specimens were handled in accordance with the FDA-approved protocol. The evaluation involved the blinded assessment of samples for both IHC and DISH, scoring 20 nuclei for the the quantity of HER2 and centromere 17 signals within every cell. The HER2/centromere 17 probe signal ratio was then calculated through the analysis of 20 cells.

Statistical analysis: Statistical analysis of the data was conducted using a computer software program designed for Windows, employing Statistical Packages for Social Sciences (SPSS-22). Continuous variables were presented with mean and standard deviation, while categorical variables were demonstrated in frequencies and percentages. Categorical variables underwent analysis utilizing the Chi-Square test, demonstrated through cross-tabulation. For continuous variables, the ANOVA test was applied, illustrating results with mean and standard deviation. Statistical significance was established at a p-value <0.05.

Results:

The mean age was 48.6±11.6 years varied from 28 to 75 years. All 55(100%) patients were female and the mean tumour size was 3.5±1.6 cm. All (100%) patients had invasive ductal breast carcinoma (NOS). Grade II and grade III tumour were 36(65.5%) and 19(34.5%) respectively. Lymphovascular invasion (LVI) was seen in 34(61.8%) of the patients. Positive for estrogen receptor in 25(45.5%) and progesterone receptor positive was found in 20(36.4%) cases. Majority 35(63.6%) patients showed lymph node metastases in N1-N3 groups. The vast majority 45(81.8%) patients had 2+ HER 2 IHC, 8(14.5%) had 3+ HER 2 IHC and 2(3.6%) had 1+ HER 2 IHC. DISH assay techniques demonstrated amplification of the HER2 gene in 40(72.7%) cases. The mean total Her-2 count was found 157.8±99.0 and the mean total Chr-17 count was noted 39.4±11.9. The mean HER 2/Chr 17 ratio was 3.7±3.0 and almost three fourth 39(70.9%) patients had ≥4 average HER2 number/nucleus. Patients were in 5th decade and above, had tumour size >2 cm and tumour grade III showed DISH positivity more. Tumour had lymphovascular invasion also showed DISH positivity more than LVI negative cases. Metastatic breast cancer showed high copy DISH positivity (>5.0 HER 2/Chr 17 ratio) and primary breast cancer showed low copy DISH positivity (2.1-5.0 HER 2/Chr 17 ratio). More than two third 31(68%) immunohistochemically equivocal cases had ≥4 average HER 2 per/nucleus with >2.0 HER 2/Chr17 ratio. HER 2 IHC 1+ had (100.0%) concordance with DISH. IHC 2+ had (73.3%) concordance and IHC 3+ had (87.5%) concordance with DISH. On the contrary, the discordance rate by IHC was not sky-high (26.7%) in IHC 2+ cases. In this current study, the sensitivity of IHC were calculated 97.6%, accuracy 76.4%, positive predictive value 76.9% and negative predictive values 66.7% with Dual In Situ Hybridization assumed as the benchmark testing method.

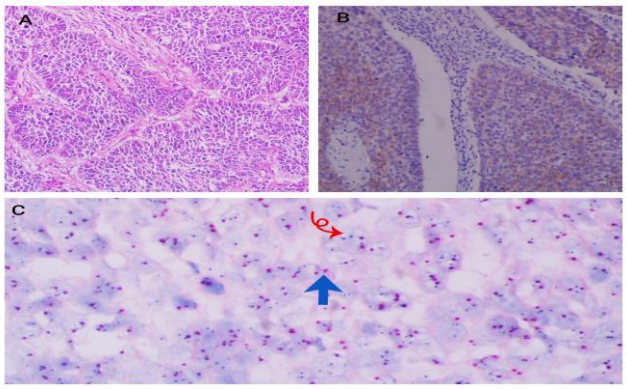

Figure 1: Photomicrographs showing: A: Invasive ductal breast carcinoma (Stain: H&E x 400), B: HER2/neu immunohistochemistry (Score 1+ HER-2 IHC x 400)**, C:** Non amplified HER2/neu gene in IHC 1+ case. HER2/neu gene status (black dot) marked by red arrow and CEN- 17 (red dot) marked by blue arrow (DISH x 600). (Case -5)

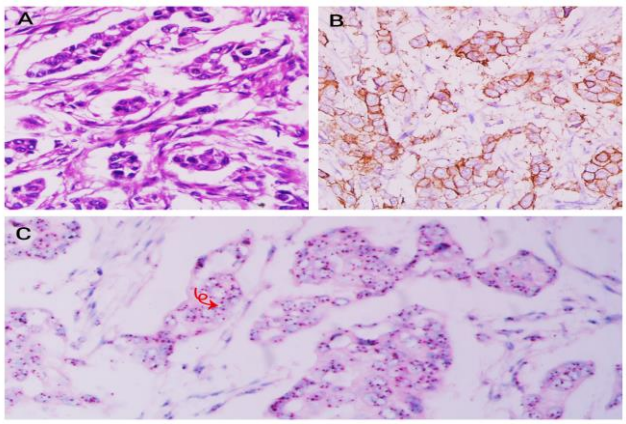

Figure 2: Photomicrographs showing: A: Invasive ductal breast carcinoma (Stain: H&E x 400), B: HER2/neu immunohistochemistry (Score 2+ HER-2 IHC x 400), **C:** Non amplified HER2/neu gene pointed by red arrow in IHC 2+ case (DISH x 600). (Case -8)

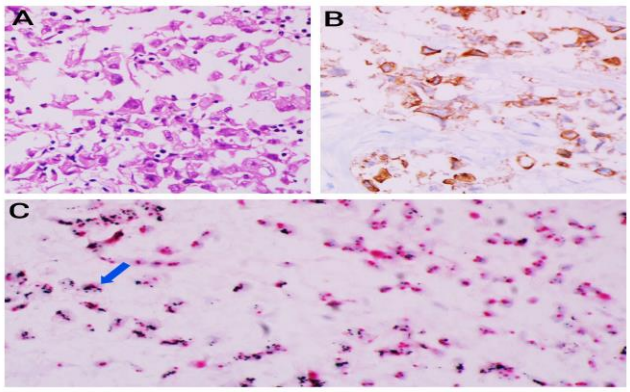

Figure 3: Photomicrographs showing: A: Invasive ductal breast carcinoma (Stain: H&E x 400), B: HER2/neu immunohistochemistry (Score 2+ HER2 IHC x 400), **C:** Amplified HER2/neu gene pointed by blue arrow in IHC 2+ case (DISH x 600). (Case -11)

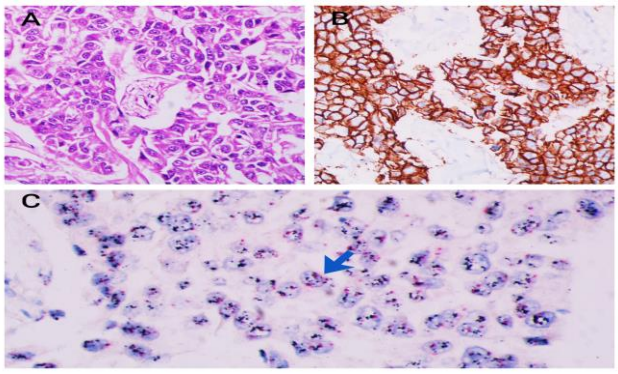

Figure 4: Photomicrographs showing: A: Invasive ductal breast carcinoma (Stain: H&E x 400), B: HER2/neu immunohistochemistry (Score 3+ HER2 IHC x 400), **C:** Amplified HER2/neu gene marked by blue arrow in IHC 3+ case (DISH x 600). (Case -13)

Table I: Arrangements of the study participants by age (n=55)

Age (in years)	Number of patients	Percentage
≤30	4	7.3
31-40	12	21.8
41-50	17	30.9
51-60	12	21.8
>60	10	18.2
Mean±SD	48.6±11.6	
Range (min, max)	28, 75	

Table II: Arrangements of the study participants by tumour size (n=55)

Tumour status	Number of patients	Percentage
Tumour size (cm)		
≤2.0	10	18.1
2.1-5.0	36	65.5
>5.0	9	16.4
Mean±SD	3.5±1.6	
Range (min, max)	1.6, 9.0	

Chart I: Arrangements of the study participants by HER2 IHC

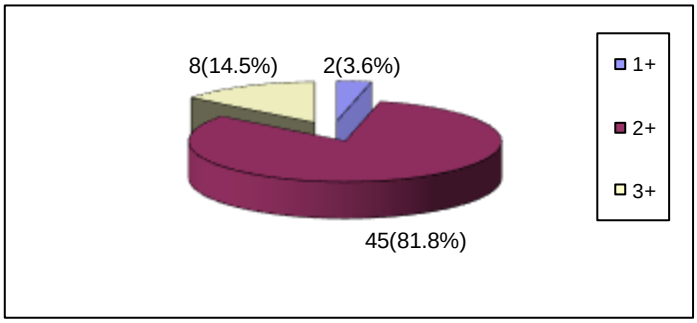

Table III: Concordance analysis of HER2 by IHC and DISH method (n=55)

IHC	Total	HER 2 DISH assay			
		Number of patients		Concordance	Discordance
		Not Amplified (n=15)	Amplified (n=40)	%	(%)
1+	2	2	0	100.0	0
2+	45	12	33	73.3	26.7
3+	8	1	7	87.5	12.5

Table IV: HER2 gene amplification specified by HER2/Chr17 ratio and mean absolute HER2 gene copy number in 55 patients

HER2 IHC	Average HER2 per/nucleus	HER2/Chr17 ratio						p value
		<2.0 (n=16)		2.1-5.0 (n=19)		>5.0 (n=20)		
		n	%	n	%	n	%	
1+ (n=2)	<4 (negative)	2	100.0	0	0.0	0	0.0	-
	≥4 (positive)	0	0.0	0	0.0	0	0.0	
2+ (n=45)	<4 (negative)	12	26.7	1	2.2	0	0.0	0.001[s]
	≥4 (positive)	1	2.2	15	33.3	16	35.6	
3+ (n=8)	<4 (negative)	1	12.5	0	0.0	0	0.0	0.018[s]
	≥4 (positive)	0	0.0	3	37.5	4	50.0	

s= Significant, p value estimated utilizing chi square test.
Table IX shows association between average HER2 number with HER2/Chr17 ratio and the disagreement was significant statistically (p>0.05) amidst three groups.

Table V: Statistical analysis of IHC cases (n=55)

IHC	DISH		Total
	Positive (n=41)	Negative (n=14)	
Positive	40 (True positive)	12 (False positive)	52
Negative	1 (False negative)	2 (True negative)	3

Table VI: Sensitivity, accuracy, positive and negative predictive values of IHC (n=55) With Dual in Situ Hybridization assumed as the ideal assay technique.

Validity test	Percentage
Sensitivity	97.6
Accuracy	76.4
Positive predictive value	76.9
Negative predictive value	66.7

DISCUSSION

Breast cancer ranks as the most prevalent malignancy in females, with approximately 10% to 20% of cases presenting as invasive ductal carcinomas displaying either increased HER2 protein expression or gene amplification. Precise evaluation of HER2 amplification or expression status is crucial in identifying cases eligible for HER2-targeted drug therapy. Medical trials implementing HER2-targeted drug treatment in adjuvant nor metastatic settings utilized a combination of immunohistochemistry (IHC) along with fluorescent in situ hybridization (FISH) for determining HER2 expression status and selecting cases for enlistment and medical care. In our country, Dual In Situ Hybridization (DISH), a substitute to both FISH and IHC, is now accessible.

In the context of this study, it was noted that the largest proportion, comprising 17 patients (30.9%), fell inside the age limits of 41-50 years. The mean age was 48.6±11.6 years along with ranged between 28-75 years. Fattahi et al[9] and Ozdemir et al[10] found the mean age was 51.5±11.8 years and 50.0 years respectively, which is similar with the current research.

In this current research paper, it was found that more than two third (65.5%) patient's tumour size was in between 2.1-5.0 cm. Haroon et al.[11] and Nishimura et al.[12] noticed that major part of the tumours were in the range of 2-5 cm and 2.5-9.5 cm each, that aligns with the current research.

In this present research, it was noticed that all patients had invasive ductal carcinoma (NOS). Kilickap et al.[13] found invasive ductal carcinoma 124(76%) and invasive lobular carcinoma 14(9%), which differ with the present study, may be due to inclusion criteria of the present study.

In this study, 36(65.5%) of the cases were tumour grade II and 19(34.5%) cases were tumour grade III. Wang et al.[14] study showed that Grade I tumor was 13.6%, grade II 42.7% and grade III 40.0%, which differ with the current study due to inclusion criteria.

In this present research, it was noted that lymphovascular invasion (LVI) was present in almost two third 34(61.8%) patients. Peritumoral lymph vessel and vascular invasion (LVI) have been shown to carry prognosticating significance towards both locoregional and distant recurrence. After two decades of follow-up, Rosen et al[15] observed an interrelation among lymphovascular invasion and the risk of recurrence as well as death. Particularly, the recurrence rate in case of women along with LVI-positive stage I disease was 38.0% and 22.0% for women along with LVI-negative disease. Lymphatic vessel and vascular invasion indeed hold prognosticating signification and are basically utilized to guide valuable decisions for lymph node-negative cases with borderline tomor sizes.

In this current study, majority 35(63.6%) patients showed lymph node metastases in N1-N3 groups. Haroon et al.[11] showed lymph node metastases 62.8% in N_1-N_3 groups. This finding aligns closely with the current research. In this present research, it was noticed that majority 45(81.8%) patients had 2+ HER 2 IHC, 8(14.5%) patients had 3+ HER 2 IHC and 2(3.6%) patients had 1+ HER 2 IHC.

In this present study, it was noted that DISH assay showed amplification of the HER2 gene in almost three fourth 40(72.7%) cases. Panjwani et al.[16] tested 175 instances for HER2 gene amplification, 61.71% cases were amplified by FISH. Gao et al.[17] reported HER2 amplification by DISH in 100% of 20 IHC 3+ instances in their academic pursuits indicates that cases with unequivocally positive results are not overlooked or missed by DISH. Kuo et al.[18] noticed that 53% of IHC 2+ instances and 83% of the IHC 3+ instances were HER-2 FISH positive. Considerably high discordance rates by IHC were also found in this study, with 46.7% in IHC 2+ and 16.7% in IHC 3+ cases.

In this present research paper, it was found that more than one third 20(36.4%) patients had HER2/Chr17 ratio ≥5.0. Almost three fourth (70.9%) patients had ≥4 average HER2 number/nucleus.

In this present study, one case of IHC score of 3+ did not display gene amplification by DISH due to one of the following reasons such as over staining in Immunohistochemistry, the sample was misread as 3+ in HER2 IHC or gene amplification beneath the detection level of the DISH method. Panjwani et al.[16] identified three cases with an IHC score of 3+ that did not exhibit gene amplification according to FISH. In two out of three cases, polysomy 17 was observed, and the false positivity in the other case could be attributed to reasons such as too much antigen retrieval, isolated-copy overexpression of the HER2 gene on the mRNA transcriptional level or expression beyond except actual gene amplification, or gene amplification beneath the detection threshold of the FISH method.

About the concordance study of HER 2 by IHC and DISH method in this current research, it was noticed that IHC 1+ had (100.0%) concordance. IHC 2+ had (73.3%) concordance and IHC 3+ had (87.5%) concordance. Panjwani et al.[16] tested 175 instances for HER2 gene amplification, 108 instances (61.71%) were amplified by FISH. The IHC 3+ instances (93.9%) and IHC-negative instances (85.96%) demonstrated a strong correlation with FISH results, whereas a notable 66.6% of cases in the IHC 2+ group exhibited gene amplification.

Contrarily, the discordance rates by IHC were 26.7% in IHC 2+ and 12.5% in IHC 3+ cases in this present research. Kuo et al.[18] found the discordance rates by IHC were high up (46.7% in IHC 2+, 16.7% in IHC 3+) in comparison to FISH.

In this present research, it was observed that in immunohisto-chemically negative (1+) cases, HER 2/Chr 17 DISH ratio were <2.0. Similarly among 45 immunohisto-chemically equivocal (2+) cases, HER 2/Chr 17 DISH ratio were >2 in 32(70%) cases. Immunohistochemically positive (3+) cases showed high copy DISH positivity (>5.0 HER 2/Chr 17 DISH) in maximum cases.

In this present research, it was noticed that the mean total HER2 count was found 157.8±99.0 and mean total Chr-17 count was noted 39.4±11.9. The mean HER 2 as well as Chr 17 per nucleus and HER 2/Chr 17 ratio were found more in IHC 2+ and IHC 3+ cases than immunohistochemically negative cases.

For statistical evaluation of IHC, 40 true positive cases, 12 false positive cases, 2 true negative cases along with 1 false negative case were identified. In this current research, it was noted that the validity test of IHC were performed by calculating sensitivity 97.6%, accuracy 76.4%, positive predictive value76.9% and negative predictive values 66.7%. Panjwani et al.[16] documented that the comparison between the two groups demonstrated a notably high-level sensitivity of 93 percent in the amalgamated group of IHC 2+ and IHC 3+ cases, as opposed to the 71.0% observed in IHC 3+ alone. Panjwani et al.[16] study found sensitivity 93.0%, positive predictive value 88.0%, negative predictive value 88.0% and accuracy 87.7%. Tsuda et al.[19] reported a significantly higher sensitivity of 97 percent when the IHC 3+ cases were evaluated.

Conclusion: HER2/neu status stands out as one of the most crucial prognosticating and prophetic factors, representing a treatment target for the anti-HER2 antibody trastuzumab (Herceptin). However, the assessment of HER2/neu expression status via IHC can pose challenges and inaccuracies, particularly in patients along with an IHC score of 2+. The vast majority of the patients were in 5th decade and above, and invasive ductal cell carcinoma was observed in my study patients. Tumour grade II and Lymphovascular invasion were more common in this study. The sensitivity of IHC were calculated 97.6%, accuracy 76.4%, positive predictive value 76.9% and negative predictive values 66.7% with Dual in Situ Hybridization was considered as the preferred testing method. DISH assay showed amplification of the HER2 gene in almost three fourth cases of current study. Based on the present results, DISH emerges as a valuable, straightforward and standardized reproducible technique for identifying HER2/neu gene amplification, particularly in patients along with borderline (2+) immunohistochemistry scores. Those specific patients should get advantages from HER2-targeted drug therapy such as Trastuzumab.

This study has few limitations include small-scale sample size, only one centre study and short duration of study period.

Recommendations: Subsequent research can be conducted by incorporating a substantial number of participants.

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