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Original Research

Elevated FISH Her-2/neu Amplification in ER Positive, IHC Her-2 Equivocal Invasive Breast Cancer Patients of North-East India

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Background: Breast cancer management of Her-2 equivocal breast cancer patients requires assessment by fluorescent in situ hybridization (FISH) for Her-2/neu amplification. Assessment of FISH Her-2/neu amplification status in IHC Her-2 equivocal invasive breast cancer patients of North-East India.

Methods: One hundred sixty-two (n=162) IHC Her-2 equivocal (2+) breast cancer patients were analysed by FISH for Her-2/neu amplification using PathVysion Her-2/neu DNA probe kit. The interpretation of FISH Her-2/neu amplification was based on ASCO/CAP 2018 guidelines.

Results: FISH Her-2/neu amplification was detected in 59.26% (n=96) patients and 40.74% (n=66) patients had no amplification. There were 160 female and 2 male patients. ER-positive patients were 68.51% (n=111) and PR-positive were 54.93% (n=89). “ER+/PR+” status was found in 54.93% (n=89) patients whereas “ER-/PR-’’ in 31.48% (n=51) patients. Significant association was observed between histological grade and FISH Her-2/neu amplification (p=0.036). ER-positive patients had also shown significant association with FISH Her-2/neu amplification (p=0.039). ER+/PR+ patients had shown a trend for higher likelihood of FISH

Her-2/neu amplification (p=0.053). There were no significant association observed between FISH Her-2/neu amplification with age (p=0.625), lymph node positivity (p=0.642), laterality (p=0.367), histopathology (p=0.226) and PR-positivity (p=0.199).

Conclusion: FISH Her-2/neu amplification in IHC Her-2 equivocal breast cancer patients of North-East India was found to be significantly associated with ER-positive status and histological grade. ER+/PR+ patients have also an enhanced likelihood of FISH Her-2/neu amplification.

Introduction

Globally among women, breast cancer (BC) remains one of the most challenging health concern in developing as well as developed countries [1]. In India, being the most common malignancy breast cancer accounts for 13.5% of all cancer cases and 10.6% of all cancer deaths [2, 3]. Age adjusted incidence rate (AAR) per 100000 population among females for breast cancer in India range from 7.0- 48.0. In Northeast India, probability of developing breast cancer over a lifetime in females is as follows: Kamrup Urban (1 in 32), Mizoram state (1 in 43), Pasighat (1 in 55), Dibrugarh district (1 in 62), Cachar district (1 in 73), West Arunachal (1 in 92), Manipur (1 in 95), Nagaland (1 in 109), Tripura state (1 in 123) and Meghalaya (1 in 142) [4].

Human epidermal growth factor receptor 2 (Her- 2), belonging to the family of epidermal growth factor receptors (EGFR), is a transmembrane tyrosine kinase receptor [5]. The protein is located on the long arm of chromosome 17 (17q12-21.32) and encoded by the Her-2 (ERBB2) gene [6]. Amplification and overexpression of this oncogene induces proliferation and antiapoptotic as well as tumorigenic growth in human mammary epithelial cells [7]. About 15-20% patients with invasive breast cancer have Her-2 protein overexpression and/or Her- 2 gene amplification [8]. The advent of Her-2 targeted therapy like Trastuzumab has dramatically improved clinical outcomes in Her-2 positive breast cancer [9]. Thus, accurate diagnostic testing for Her-2 is an important front-line investigation in individuals with invasive breast cancer. The American Society of Clinical Oncology/College of American pathologists (ASCO/CAP) guidelines 2018, recommended Her-2/neu testing by immunohistochemistry (IHC), and when equivocal (2+), the same needs to be assessed by in situ hybridization (ISH) techniques like fluorescence in situ hybridization (FISH) [10].

The ASCO/CAP 2018 guidelines define four groups of IHC results, viz., 0 (negative), 1+ (negative), 2+ (equivocal), and 3+ (positive), based on criteria for staining pattern, intensity, and the percentage of tumor cells stained [10]. Based on the Her-2/CEP17 ratio and average Her-2 copy number per cell in relation with the immunohistochemical results, the guidelines recommended criteria for FISH results to establish 5 dual probe ISH groups [11].

IHC Her-2/neu equivocal (2+) breast cancer patients with gene amplification status by FISH have shown wide range variation in various geographical regions as follows: India (14% to 71%) [12-19]; China (29% to 70%) [20-23]; Germany (14% to 26%) [24-26]; USA (8%,64%) [27, 28]; Thailand (37.5%) [29]; Egypt (18%) [30]; Hungary (38%) [31]; Netherland(24%) [32]; Denmark (48%) [33]; Taiwan (53%) [34]; Australia (0%) [35]; Iran (31.1%) [36]. Among these studies, only 7 studies have more than 100 breast cancer patients with Her-2/neu IHC Equivocal (2+) status and their corresponding FISH evaluation [13, 15, 16, 20-22, 26].

There is lack of data about Her-2/neu FISH status in Her-2/neu IHC Equivocal (2+) in breast cancer patients of North-East India. This study aims to evaluate Her-2/neu gene amplification status by FISH in IHC equivocal (2+) invasive breast cancer patients from North-East India alongwith clinicopathological characteristics.

Materials and Methods

A total of 162 patients diagnosed with invasive breast cancer and IHC Her-2/neu equivocal (2+) status were enrolled from January 2021 to June 2023 and were analysed for Her-2/neu amplification by FISH. Patients with benign tumors of the breast, other histopathological subtypes of malignant breast tumors, and incomplete clinicopathological information were excluded from the study. The patient's demographic and clinicopathological characteristics of the tumor were recorded. This study was approved by Institutional Ethics committee (.

Immunohistochemistry (IHC): IHC staining was done on 4-5µm thickness sections of freshly cut formalin-fixed, paraffin-embedded tumor tissue from the same specimen blocks that were used for FISH Her-2/neu testing.Ventana BenchMark XT (Roche diagnostics) protocol for Her-2 IHC was followed as per manufacturer's instruction. The details of the antibodies used on the Ventana BenchMark XT (Roche diagnostics) were as follows:

Antibodies Clone

ER SP1 (rabbit monoclonal)

PR 1E2 (rabbit monoclonal)

HER2 4B5 (rabbit monoclonal)

IHC scoring was performed by two independent pathologists as per the ASCO/CAP 2018 guidelines [11]. Fluorescence in situ hybridization (FISH): PathVysion Her-2 DNA Probe Kit (Abbott, USA) was used to detect Her-2/neu gene amplification as per the manufacturer's protocol. In brief, the probes were pre-mixed and pre-denatured in hybridization buffer. Formalin-fixed, paraffin-embedded tissue specimens were placed on slides. The DNA was denatured to single-stranded form and then allowed to hybridize with the PathVysion probes. After that, the unbound probe was removed by series of washes and the nuclei were counterstained with DAPI (4, 6 diamidino-2-phenylindole). FISH slides were visualized at 100X using fully motorized BX61 fluorescence microscope (Olympus, Japan) with appropriate excitation and emission filters allowing visualization of the intense orange and green, fluorescent signals. Enumeration of the orange Locus Specific Identifier (LSI) Her-2/neu and green Chromosome Enumeration Probe (CEP) 17 signals was conducted by microscopic examination of the nucleus, which yielded a ratio of the Her-2/neu gene to chromosome 17 copy number. ASCO/CAP 2018 guidelines were followed for Her-2/neu FISH scoring and interpretation by two independent observers [11].

Statistical Analysis

Chi square test and Fisher's exact test were applied to observe the association between clinicopathological features, hormone receptors and FISH Her-2/neu amplification respectively. All the statistical analyses were performed in SPSS 29.0 software (IBM Corp, NY, USA). A p-value < 0.05 was considered to be statistically significant.

Results

A total of 162 IHC Her-2 equivocal (2+) invasive BC patient's clinicopathological data were analyzed (Figure 1). There were 160 female and 2 male patients. The median age of the BC patients was 48 years, range=23 – 83 years. 61.11% (n=99) BC patients were <50 years and 38.88% (n=63) were > 50 years. Based on the Nottingham classification, 59.87% (n=97) BC patients had

histological grade II followed by 38.27% (n=62) grade III and 1.86% (n=03) grade I. Lymph node-positive outcome was detected in 35.18% (n=57) patients. 51.23% of BC patients (n=83) had cancer in the right breast and 48.77% (n=79) in the left breast. All patients (n=161) were histopathologically invasive ductal carcinoma (IDC) except one which was invasive lobular carcinoma. Hormone receptor status was as follows : 68.51% (n=111) were ER+ and 54.93% (n=89) were PR+. “ER+/PR+” status was found in 54.93% (n=89) patients whereas “ER-/ PR-’’ in 31.48% (n=51) BC patients (Table 1).

Characteristics		Number of patients	Percentage
Age	≤50	99	61.11
	>50	63	38.88
Gender	Male	2	1.23
	Female	160	98.77
Grade	Grade I	3	1.86
	Grade II	97	59.87
	Grade III	62	38.27
Lymph node involvement	Present	57	35.18
	Absent	35	21.06
	No information	70	43.2
Laterality	Left	79	48.77
	Right	83	51.23
Histology	Invasive ductal carcinoma	161	99.38
	Invasive lobular carcinoma	1	0.62
ER	Positive	111	68.51
	Negative	51	31.48
PR	Positive	89	54.93
	Negative	73	45.07
Hormone receptor status	ER+PR+	89	54.93
	ER-PR-	51	31.48
	ER-PR+	0	0
	ER+PR-	22	13.58

Table 1. Data on Clinicopathological Characteristics in Her-2 equivocal Invasive Breast Cancer Patients (n=162).

Her-2/neu amplification by FISH was detected in 59.26% (n=96) patients whereas 40.74% (n=66) patients had no amplification (Table 2), (Figure 1).

IHC HER 2	FISH Her-2/neu		Segment of Amplified	Segment of Non-Amplified
	Amplified	Non-Amplified		
2+	96	66	59.26%	40.74%

Table 2. Proportion of FISH Her-2/neu in terms of IHC HER 2.

Figure 1. IHC and FISH Images of Breast Cancer Patients. A. IHC Her-2 (2+ Score); B. FISH Her-2 /neo (non amplified); C. FISH Her-2/neo (amplified).

A significant association was observed between histological grade and FISH Her-2/neu amplification (p=0.036). There was no significant association found between FISH Her-2/neu amplification status with age (p=0.625), lymph node positivity (p=0.642), laterality (p=0.367), and histopathology (p=0.226) (Table 3).

Clinicopathological Features		FISH Her-2/neu		p-value
		Amplified (%)	Non-Amplified (%)	
Age	≤ 50	57 (59.37)	42 (63.63)	0.625
	> 50	39 (40.62)	24 (36.36)	
Grade	Grade I	00 (0.00)	03 (4.55)	
	Grade II	63 (65.63)	34 (51.52)	0.036*
	Grade III	33 (34.37)	29 (43.93)	
Lymph Node Involvement	Present	31 (32.29)	26 (39.40)	0.642
	Absent	22 (22.91)	13 (19.69)	
	No Information	43 (44.80)	27 (40.91)	
Laterality	Left	44 (45.84)	35 (53.03)	0.367
	Right	52 (54.16)	31 (46.97)	
Histology	Invasive ductal carcinoma	96 (100)	65 (98.49)	0.226
	Invasive lobular carcinoma	00 (00.00)	01 (1.51)	

Table 3. Association between Clinicopathological Features and FISH Her-2/neu Outcome.

*Statistically Significant

ER-positive patients had shown significant association with FISH Her-2/neu amplification (p=0.039). ER and PR expression was higher in FISH Her-2/neu amplified tumors compared to non-amplified tumors. Although ER+/ PR+ patients had higher FISH Her-2/neu amplification but failed to achieve significant association (p=0.053). PR-positive cases had also not shown any significant association with FISH Her-2/neu amplification (p=0.199) (Table 4).

Hormone Receptor		FISH Her-2/neu		p-value
		Amplified	Non-Amplified	
ER	Positive	72	39	
	Negative	24	27	0.039*
PR	Positive	57	32	
	Negative	39	34	0.199
Hormone receptor status	ER+PR+	57	32	
(Both positive/ Both Negative)	ER-PR-	24	27	0.053
Hormone receptor status	ER-PR+	0	0	1
(Either negative/either positive)	ER+PR-	15	7	

Table 4. Association between Hormone Receptor and FISH Her-2/neu Outcome.

*Statistically Significant

Discussion

Reliable laboratory data in evaluating Her-2 status is essential. Her-2 status studied at the levels of DNA using FISH and protein using IHC are the two most accessible and feasible methods used in clinical diagnosis [1]. Breast cancer is the most common malignancy among women in India. The

treatment and prognosis of patients with breast cancer depend on various factors, such as grade, histology, hormone receptor status, and Her-2/neu status. Her-2/neu status determination is necessary in breast cancer patients for Her-2 targeted therapy (e.g.trastuzumab) [37, 38].

In our study of IHC Her-2 equivocal (2+) BC patients, FISH Her-2/neu amplification was detected in 59.26% (96/162) whereas it was non-amplified in 40.74% (66/162). Previous studies from India have shown higher Her-2/neu amplification in IHC Her-2 equivocal BC patients: 54% (72/134); 66.6% (24/36); 70% (7/10); 71% (20/28) [16-19]. However, other Indian studies also reported lower coincidence rate of IHC 2+/FISH Her-2/neu amplified BC cases – 14.3% (1/7); 24.4% (31/127); 25% (17/68) and 28.1 % (68/242) [12-15]. Except for three studies, rest all Indian studies have quite small sample sizes of IHC Her-2 (2+) BC patients. These studies are from various regions of India except North-East. Therefore, variations in the coincidence rate of IHC 2+/FISH Her-2/neu amplified BC patients may be attributed to the patient's ethnic, genetic, geographical and etiological characteristics etc. We had followed the 2018 ASCO/CAP guidelines for Her-2/neu FISH scoring and interpretation whereas most of the previous studies cited by us may not have followed the 2018 guidelines and this could also have contributed to variation in FISH Her-2/neu amplification outcomes. The differences in FISH Her-2/neu may also arise due to pre-analytical condition, variation in probes and individual interpretative reporting [23, 39].

Globally FISH Her-2/neu amplification in IHC equivocal (2+) BC patients have also shown wide range variation: China (29% to 70%); Germany (14% to 26%); USA (8%, 64%); Thailand (37.5%); Egypt (18%); Hungary (38%); Netherland (24%); Denmark (48%); Taiwan (53%); Australia (0%); Iran (31.1%) [20-36].

In the Asian population, BC has a younger age at presentation i.e. 40-50 years, a decade earlier as compared to Western countries [40]. In our study, median age of breast cancer patients was 48 years. Earlier studies found the median age of Her-2/neu IHC equivocal BC patients as follows: 50 years & 52 years (India) [16,14], 42.5 years (China) [20] and 63 years (Canada) [41]. We also found that 61.11% patients (n=99) were < 50 years while the rest 38.88% (n=63) were > 50 years. The differences in the median age may be attributed to the population characteristics and variations in risk factors.

We found histological grade II in 59.87% (n=97) of the BC patients as the most prevalent histological grade. Murthy et al (44/68, 70.5%) and Zhang et al (321/478, 67.2%) had also found histological grade II as the most prominent grade in Her-2/neu IHC (2+) BC patients [14, 20]. We also observed significant association between FISH Her-2/neu amplification and histological grade (p=0.036). Panjwani et al also found a significant association between histological grading and FISH Her-2/ neu amplification [17]. However, Mostafa et al found no correlation between histological grade and FISH Her-2/ neu amplification in IHC (2+) BC patients [30]. These differences in histological grade and FISH Her-2/neu amplification may be attributed to the differences in the biological characteristics of the tumor, methods used for IHC & FISH and patient cohort size.

Our data had shown "ER+/PR+" status (54.93%) as more predominant compared to "ER-/PR- '' (31.48%) which is also in concordance with Zhang et al report ("ER+/PR+" = 60.2%; "ER-/PR-''= 28.4%) [20]. We also observed that ER and PR expression were higher in FISH Her- 2/neu amplified tumors compared to FISH Her-2/neu non-amplified tumors. We found significant association between ER-positive patients and FISH Her-2/neu amplified tumors (p=0.039). However, various Indian studies reported association between Her-2/neu amplification and negative hormone receptor expression [14-17]. Her-2 amplification and ER-positive tumors co-positivity is reported to be associated with resistance to selective ER modulators like tamoxifen therapy [42]. It is postulated that Her-2 cross-talk with ER could increase the estrogen agonistic activity of tamoxifen leading to enhanced tumor growth resulting in de novo resistance to tamoxifen [43, 44]. The observed differences in hormone receptor expression status may be associated to tumor's biological profile and antibodies used for hormone receptor expression in IHC and probes used in FISH

analysis.

But, we didn't found any significant association between age (p=0.625), lymph node involvement (p=0.642), and laterality (p=0.367) with FISH Her-2/ neu amplification status. Ji et al also noted no significant difference between Her-2/neu status with respect to age and cancer location in IHC (2+) invasive breast cancer [22]. Zhang et al found no correlation between Her-2/neu expression and age (p=0.53) but in lymph node involvement, they found trends without statistical significance (p=0.072) [20].

To the best of our knowledge, this is the first study from Northeast India analysing FISH Her- 2/neu amplification in IHC Her-2 (2+) equivocal breast cancer patients. We had also observed that ER-positive patients have direct proportional relationship with FISH Her-2/neu amplification whereas other studies have reported inverse relationship with FISH Her-2/neu amplification in IHC Her-2 (2+) equivocal breast cancer patients. Limitations of our study are that it is a single tertiary cancer care centre data. Sample size was moderate.

In conclusion, we conclude that FISH Her-2/neu amplification in IHC Her-2 equivocal (2+) breast cancer patients of North-East India was found to be significantly associated with ER-positive status and histological grade. ER/PR positive breast cancer patients have an enhanced likelihood of FISH Her-2/neu amplification. To validate our current study observation, further studies with multicentre, larger sample cohort of Her-2 equivocal (2+) breast cancer patients will be required.

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Statement of Transparency and Principals:

• Author declares no conflict of interest

• Study was approved by Research Ethic Committee of author affiliated Institute.

• Study's data is available upon a reasonable request.

• All authors have contributed to implementation of this research.

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