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Original Research Article

Study of HER-2 Gene Amplification by Chromogenic in-situ hybridization and HER-2 Protein Amplification by IHC in Breast Cancer using Manual Tissue Microarray Sections

Nagalakshmi J.[a], Suresh R.[b], S. Archana[c], Eswari V.[d]

[a,b,c]Assistant Professor [d]Professor, Department of Pathology, Meenakshi Medical College Hospital and Research Institute, Enathur, Kanchipuram, Tamil Nadu 631552, India.

Corresponding Author:

Suresh R.,
Assistant Professor,
Department of Pathology,
Meenakshi Medical College
Hospital and Research Institute,
Enathur, Kanchipuram, Tamil
Nadu 631552, India.
E-mail:
sureshmmc04@gmail.com]

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Abstract

Aim*:* Breast cancer is the most common cancer in women affecting one in eight in their lifetime [1]. HER-2 gene is amplified in approximately 20-40% of breast cancers [2], which correlates with aggressive disease and amenable to treatment with Trastuzumab. The aim of this study is to detect amplification of HER-2 gene by chromogenic insitu hybridization (CISH) and HER-2 protein by immunohistochemistry in Manual Tissue microarray sections of breast cancer.

Materials and Methods*:* 30 confirmed breast cancer cases were included in the study in which adequate tissue material were available. The breast tissue fixed in 10% buffered formalin were routinely processed and HE stained slide obtained for confirmation of tumour tissue in the paraffin block. A total of 2 Tissue microarray blocks were made each having 15 cores each from different patients using the method described by Singh et al . Sections were taken from each microarray block for HE staining, CISH and IHC study.

Results: 20% showed HER-2 protein positivity. 23% were found to be positive for HER-2 gene. Cohens kappa calculated to find the degree of agreement between the HER-2 protein expression detected by IHC and HER-2 gene expression detected by CISH in Tissue microarray sections is 0.510, which indicates moderate agreement.

Conclusion: We conclude HER-2 expression in carcinoma breast could be studied by IHC and CISH with ease on Tissue microarray sections, a cost effective method which can be used for both research and diagnostic purposes in resource limited laboratories.

Keywords: Breast Cancer; HER-2/neu; Chromogenic Insitu Hybridization; Immunohistochemistry; Manual Tissue Microarray.

Introduction

Breast cancer is the most common cancer in women affecting one in eight women in their lifetime [1]. In India, breast cancer is the second most common cancer in women after cervical cancer. However, recent studies have indicated a changing trend with an increasing prevalence of breast cancer and a decreasing prevalence of cervical cancer [3]. Many factors are associated with the prognosis of breast cancer. HER-2 amplification is one of the

important predictive factor associated with breast cancer as it predicts response to Trastuzumab, a monoclonal antibody. HER-2 gene is amplified in approximately 20-40% of breast cancers [2].

HER-2 amplification in breast cancer is detected by different methods like IHC, FISH, CISH, SISH and PCR. IHC is relatively inexpensive and easy to perform but has a number of shortcomings. The interpretation of IHC is subjective and has been found to vary among observers. FISH has been used to evaluate HER-2 gene amplification in paraffin-embedded specimens, but FISH method is expensive and requires fluorescence microscopy.

CISH is a newer and relatively lesser known technique compared to FISH. CISH is an extremely accurate and sensitive technique, in which chromosomal DNA probes are used to detect HER-2 genes using an Immunohistochemical like peroxidase reaction [4]. CISH offers three important advantages to FISH; 1) Histological details of the paraffin section are generally better appreciated using conventional light microscope 2) Fluorescent microscope is not required. 3) The probe signals are not subject to rapid fading [4].

The present study is aimed at using CISH as an alternate to FISH for detecting HER-2 gene amplification in addition to IHC study of HER-2. All these IHC and CISH studies for HER-2 amplification has been performed using Manual Tissue Microarray sections to reduce the overall cost.

Materials and Methods

Experimental Design

The present study was under taken in the Department of pathology, Meenakshi Medical College and Research Institute, Kanchipuram, Tamil Nadu, India for a period of two years from October 2015 - October 2017. 30 confirmed Ductal Breast Cancer cases both prospective and retrospective were included in the study in which adequate tissue material and clinical data were available for tumour grading and staging. The breast tissue were routinely processed and HE slide sections made. HE slides were used to study the histopathological features of tumour like type of breast cancer, grading (Nottingham modification of the Bloom Richardson system) and staging (American Joint Committee on Cancer). The same HE sections were used to mark out the tumour area in the tissue block from where the cores weres taken for manual construction of Tissue microarray.

Construction of the Tissue Microarray

Tissue microarrays were constructed manually using the method described by Singh et al [5]. HE stained sections were obtained for each case. Using a marker, the area of the tumour from which the cores would be obtained was marked on the slide (Figure 1).

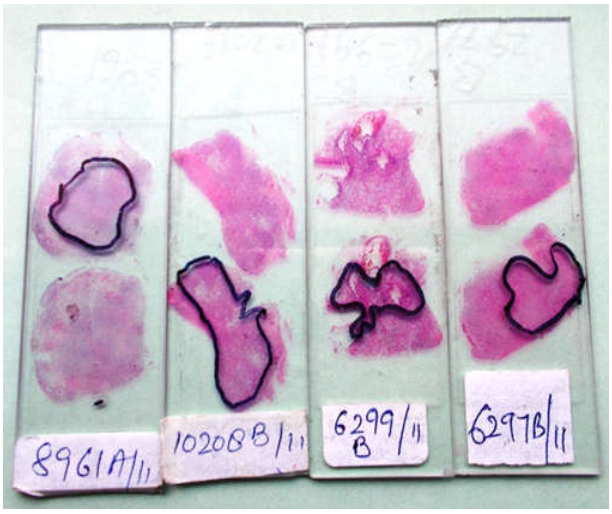

Fig. 1: The H & E stained slides from the cases to be included in the tissue microarray with the appropriate tumour areas marked

For making of the Tissue microarray sections Bone marrow aspirate needles (Salah) were used with their diameters so adjusted that the internal diameter of one was equal to the external diameter of the other. 14 and 16 gauge needles bought from the local market were used

The tips of the needles with the stylets were specially cut to make the tip flat and round and then it was slightly beveled to reduce the resistance while making holes in the paraffin block (Figure 2).

Fig. 2: The bone marrow aspirate needles (Salah) used

Blank wax blocks were taken as recipient block (Figure 3A). The cores from the tissue donor blocks were then arrayed upon it (Figure 3B, 3C). The 16 gauge needle was used to punch out a hole in the blank block. The diameter of the hole created in the blank recipient block was equal to the outer diameter of the 14 gauge needle, which was used to take tissue core from donor paraffin block

The HE stained and tumour area marked slides were placed over the donor blocks to identify the area from which the core had to be taken. Tissue core was taken out from the donor block using the 14 gauge needle and pushed into the hole using stylet. A clean glass slide was used to push the core into the hole and obtain a plain surface.

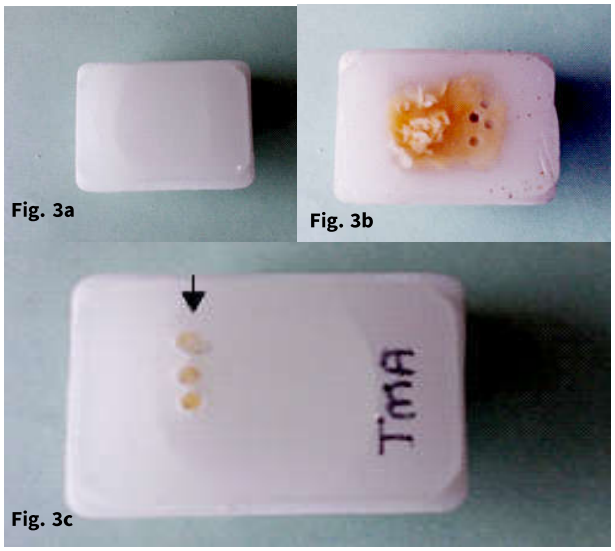

Fig. 3A: Blank paraffin block on which cores has to be arrayed. **B:** Donor block from which tissue core taken. **C:** Microarray in making-3 cores have been arrayed (arrow)

This process was repeated again and again for all the cores that were to be arrayed. A pointer core was placed at one corner for easy orientation of the array and also as positive control/negative control. TMA block so created was then heated in an oven at 100^{0}C few minutes till the wax slightly melted and filled up any residual space in the block around the cores that had been arrayed. A worksheet was created for each array that was constructed.

Two such arrays of 15 cores each in a 3X5 pattern were constructed (Figure 4).

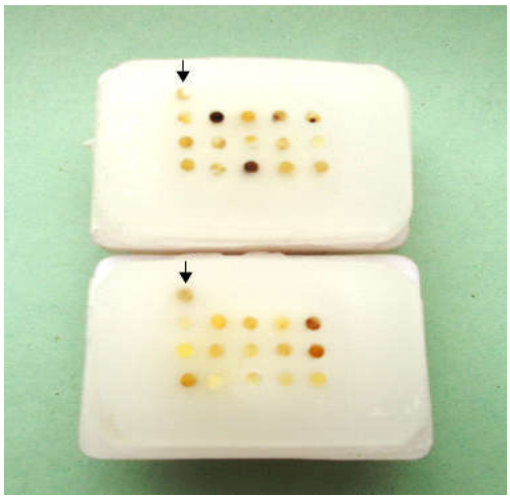

Fig. 4: Tissue microarray -3x5 with pointer core (arrow)

A detailed data base was constructed for each microarray block that was made manually

Sections from TMA block were taken for HE slide, IHC and CISH studies. polyLysine coated slides were used for IHC and CISH.

Fig. 5: Tissue microarray data grid with pointer core at one end

Pointer core(Pos/neg control)				
R141	R146	R277	R397	R403
R142	R148	R395	R399	R404
R143	R227	R396	R402	R405

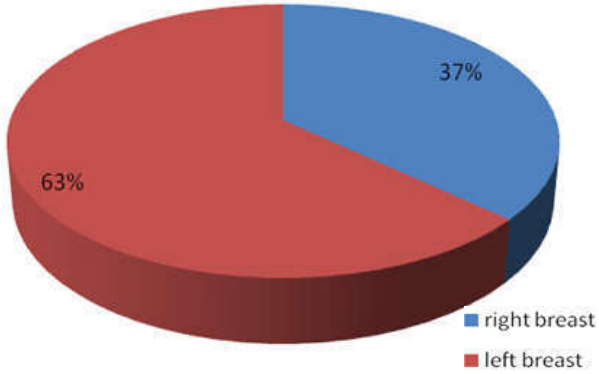

Fig. 6: Sidewise distribution of breast cancer cases (n=30)

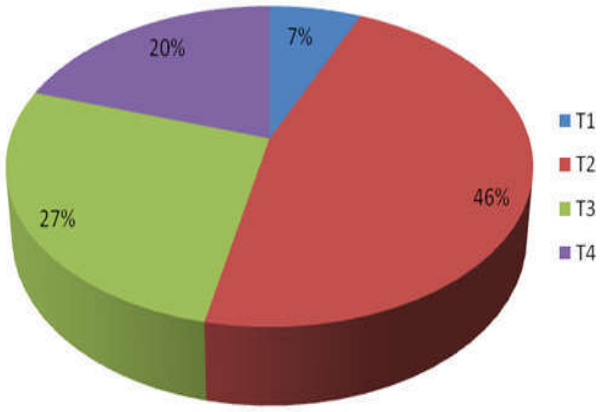

Fig. 7: T staging of the breast cancer cases as per the AJCC classification (n=30)

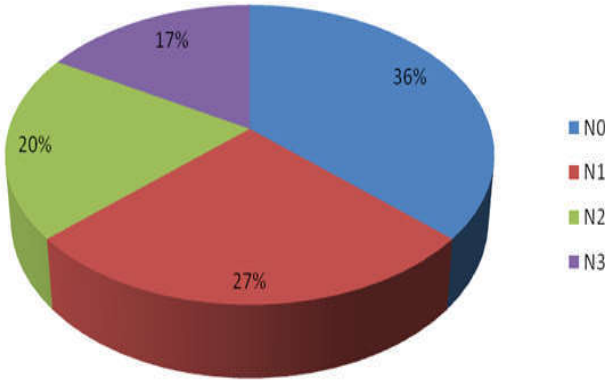

Fig. 8: N staging of the breast cancer cases as per the AJCC classification (n=30)

Immunohistochemistry

IHC performed using Monoclonal anti HER-2 (Mouse anti HER-2, clone CB-11 from Cell Marque) antibody.

Colour was developed using Diaminobenzidine Tetrahydrochloride (DAB) as a substrate. Counterstaining was done with Mayer's haematoxylin followed by dehydration, clearing and mounting.

Negative Control

Sections incubated with TRIS instead of primary monoclonal antibodies.

Positive Control

Known positive case were taken as positive control for HER2.

Immunohistochemistry Scoring System

Slides were examined at 40X magnification. Amount of positive staining was assessed as estimated percentage of positive staining cells.

HER-2: For the purpose of this study 0/1+ -negative, 2+/3+ -positive (membrane staining)[6]

- 0 - Negative. No staining or membrane staining in <10% tumour cells.
- 1+ - Negative. A faint membrane staining detected in >10% tumour cells.
- 2+ - positive. A weak to moderate complete membrane staining in >10% tumour cells.
- 3+ - Positive. A strong complete membrane staining in >10% tumour cells.

CISH

CISH for HER-2 gene amplification performed by adding 10 microlitre of DNA probe to TMA sections. Coverslip was placed over TMA section and sealed using cement glue. Slides were incubated overnight at 37 degree Celsius for hybridization.

Next day rubber cement was removed carefully and cover slips were removed by submerging in wash buffer at room temperature for 5 minutes. Slides were incubated with mouse anti-digoxinin for 30 min at room temperature and then incubated with anti-mouse HRP polymer for 30 min at room temperature after washing with PBS buffer. Color was developed using Diamino benzidine(DAB) and when appropriate color was developed, the reaction was stopped by immersing in distilled water. Slides were counter stained using Mayer hematoxylin and then dehydrated, cleared, mounted and seen under light microscopy for HER-2 gene signals.

Interpretation of Results

Visualization of signals was performed using a 40x objective. In normal diploid nuclei without gene amplification, 2 dot-shaped signals with smooth, rounded edges were seen per nucleus. Due to mitosis, additional signals were seen in a small percentage of non-neoplastic cells. In case of low gene amplifications, multiple dots or small clusters were seen in the nuclei. Small clusters means irregularly shaped signals comprising an area of up to 5 dots. As a reference, a single dot of a normal cell of the same slide was used. In case of high gene amplifications, a large number of dots or large clusters, comprising an area greater than 5 dots, were seen in the nuclei.

Negative control: Adjacent non neoplatic normal cells were taken as internal negative control

Positive control: Known case of HER-2 positive with known chromosome number was used as positive control.

Statistical Analysis

Data were analyzed using the SPSS software package, version 17.0 (SPSS Inc., Chicago, Illinois, USA). Cohen's Kappa co-efficient [7] was calculated to assess the degree of agreement between HER-2 protein amplification detected by IHC and HER-2 gene amplification detected by CISH on the Tissue microarray sections. Also, a chi square test was done to calculate p value to see any correlation between HER-2 amplification with tumour grade, size and lymph node status.

Ethical Concern

Ethical clearance was obtained from the Ethical committee meeting conducted at Meenakshi Medical College and Research Institute, Kanchipuram, Tamil Nadu, India.

Results and Observation

A total of 30 histologically confirmed cases of ductal carcinoma breast cases were included in the study. The age, side, type, size, grade, lymph node status, lymphovascular invasion and the presence or absence of in-situ component were studied in each case. The HER-2 protein status was detected by IHC and HER-2 gene status was detected by CISH in the Tissue microarray sections of breast cancer.

The age of the patients included in the study ranged from 26 to 68 years. Majority of the cases 66.66% (20/30) were seen in the age group of 31-50 years.

The right breast was involved in 11/30 (37%) cases and left breast was involved in 19/30 (63%) cases (Figure 6).

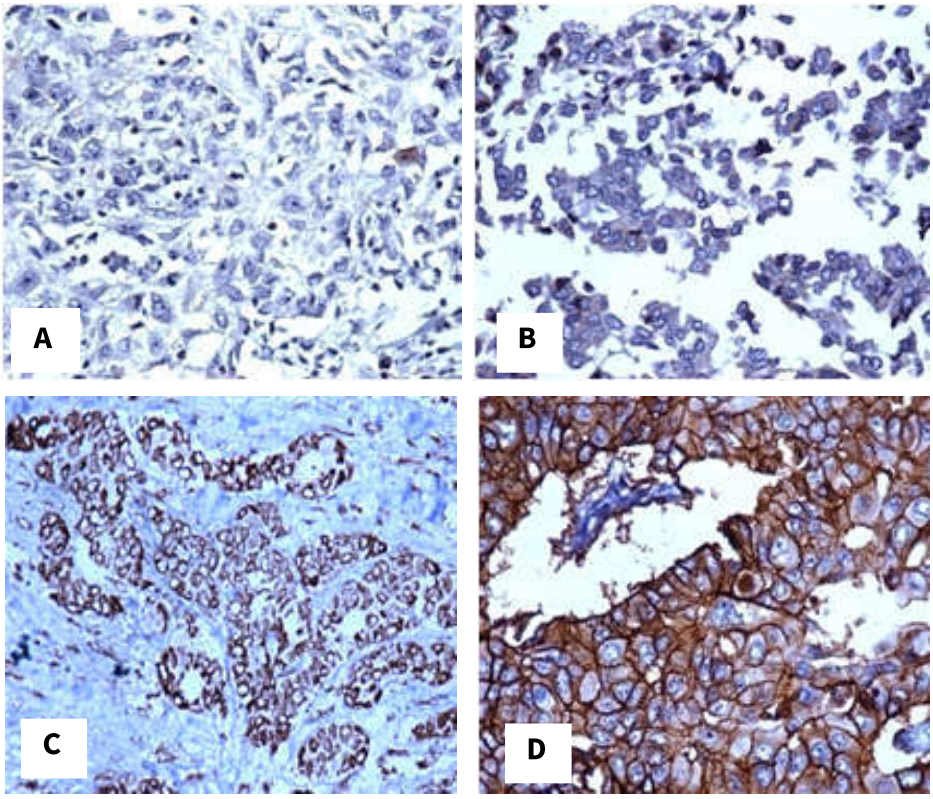

Fig. 9: Expression of HER-2 protein. **A:** 0: Negative. No staining or membrane staining in <10% tumour cells. **B:** 1+ :Negative. A faint membrane staining is detected in >10% tumour cells. The cells are only stained in part of the membrane. **C:** 2+:Positive. A weak to moderate complete membrane staining in >10% tumour cells. **D:** 3+ :Positive. A strong complete membrane staining in >10% tumour cells

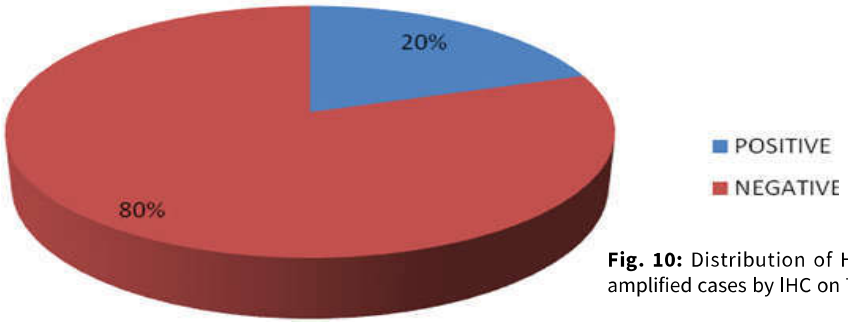

Fig. 10: Distribution of HER-2 protein amplified cases by IHC on TMA

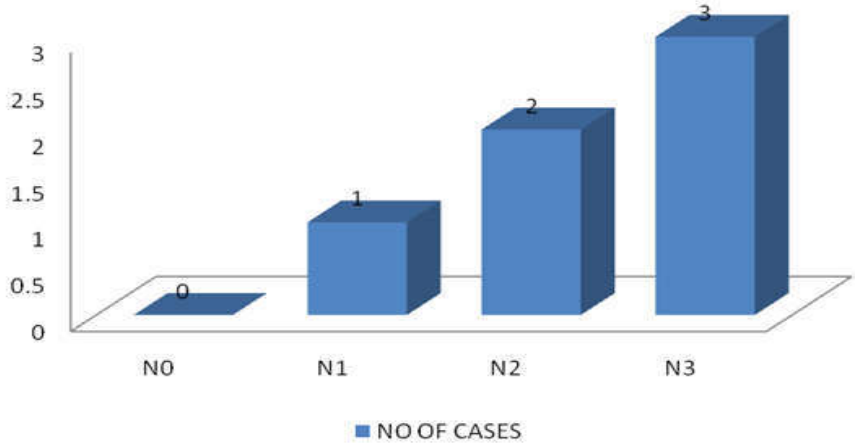

Fig. 11: Distribution of HER-2 protein positive cases as per N stage. P value <0.05

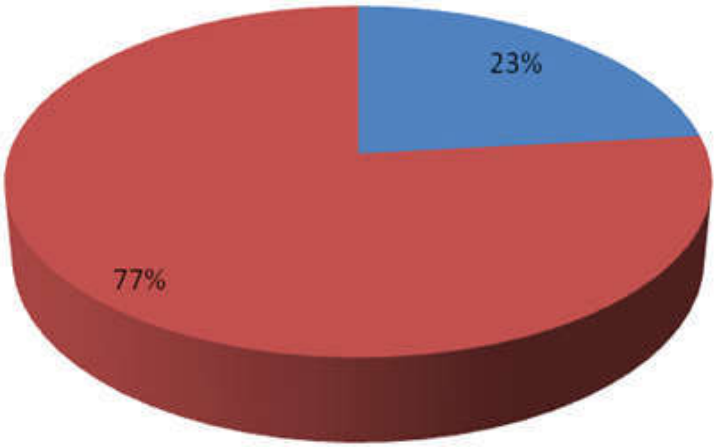

Fig. 12: Distribution of HER-2 gene over expression in breast cancer (n=30)

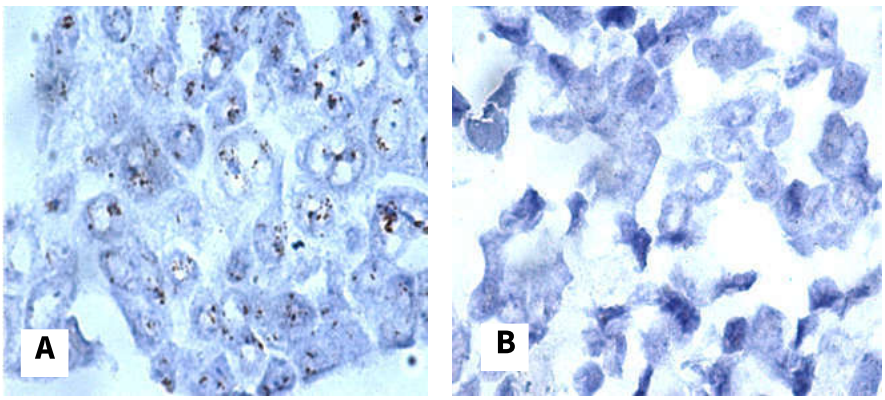

Fig. 13: HER-2 gene amplification seen as multiple dots (arrow) by CISH. 13B- Non amplified HER-2 gene by CISH

All 30 cases were of invasive ductal carcinoma (IDC) of breast. 11/30 of them were associated with ductal carcinoma insitu and 10/30 were associated with lymphovascular invasion. HER-2 protein amplified cases had increased lymphovascular invasion (66.66%) compared to 25% lymphovascular invasion seen in HER-2 protein negative cases. 50% of HER-2 protein positive cases showed associated DCIS component.

71.42% of HER-2 gene amplification cases had lymphovascular invasion and 57.14% showed associated DCIS component.

All the tumours were graded using Nottingham modification of the Bloom Richardson system [8]. 13/30 cases could not be graded as they were post chemotherapy and 1/30 case was grade I, 9/30 cases were grade II and 7/30 cases were grade III. (Table 1).

All the tumours were staged according to the AJCC classification [7]. 2/30 (7%) were in the T1 category, 14/30 (46%) were in the T2 group, 8/30 (27%) were in the T3 category and 6/30 (20%) were in the T4 category (Figure 7). 11/30 (36%) had no lymph node involvement. 8/30 (27%) were classified as N1, 6/30 (20%) were N2 and 5/30 (17%) were N3 (Figure 8). The distant metastasis could not be assessed from the mastectomy specimens received and hence all the cases were pathologically staged as Mx (metastasis cannot be assessed).

The HER-2 protein amplification by IHC were scored from 0 to 3+. (Figure 9)

6/30(20%) cases showed HER-2 protein amplification and 24/30(80%) were negative.

Distribution of HER-2 protein amplified cases were studied based on tumour size , lymph nodes and tumour grade. HER-2 expression was found to be increased in large tumours and higher grade tumours. But results were not statistically significant. However HER-2 protein expression in higher N stage group was significant with a p value of <0.05 (Figure 11).

HER-2 gene was amplified in 7/30 cases and non amplified in 23/30 cases (Figure 12).

HER-2 gene amplified cases showed large number of dots or large clusters in nucleus whereas non amplified cases showed normal signals in the nucleus (Figure 13).

Distribution of these cases in relation to grade, T stage, N stage were analysed. Cases showing HER-2 gene expression were increased in higher grade and higher stage tumours. But none of them were statistically significant.

Table 1: Distribution of breast cancer cases as per tumor grade

Grade	Cases
Not applicable (Post chemotherapy)	13
Grade I	1
Grade II	9
Grade III	7

Table 2: Degree of aggrement between HER-2 protein expression and HER-2 gene expression by kappa is 0.510, which indicates moderate agreement

HER-2 IHC		HER-2 GENE Positive	Negative	Total
	Positive	4	2	6
	Negative	3	21	24
	Total	7	23	

The main aim of this study is to detect HER-2 by CISH and IHC in manual Tissue Microarray sections and to find the degree of agreement between the HER-2 protein expression detected by IHC and HER-2gene expression detected by CISH. Cohens kappa was calculated for this. A value of <0 meant no agreement, 0 – 0.20 slight agreement, 0.21– 0.40 mild agreement, 0.41 – 0.60 moderate agreement, 0.61–0.80 substantial agreement and 0.81 to 1 perfect agreement.

Table 2 shows the degree of aggrement between HER2 ptotein and gene.

Discussion

The most important step in this study was to construct Manual Tissue Microarrays. Tissue microarrays are special paraffin blocks constructed by extracting tissue core from different paraffin donor blocks and putting these cores into a single recipient (microarray) block with pointer core for orientation and also serving as controls. We constructed 2 TMA blocks with 15 cases per block manually using the method described by Singh et al[5].

Each TMA block had 3X5 grid, with a pointer core at one corner Since automated tissue microarray machines are very costly ranging between Rs 7,74,000 ($12,000) to Rs 27,09,840 ($ 42,000) [5] we used Bone Marrow Aspirate needle set (Salah) that costs only 322 INR ($5). It is lesser than 1200 INR array set used by Singh et al. This difference in cost can be attributed to the different size needles that were used by them to obtain different core sizes. We used a single needle set of 14 and 16 gauge needles which gave a core size of 1.5mm.

The time taken to construct a microarray by this method was approximately 1 minute per core. So for a 3X5 grid, the average time taken was 15 minutes. This was comparable to the time taken by Singh et al [5].

There were certain problems that were encountered during manual TMA construction. The recipient blocks can crack and break at the time of punching the hole. This is even more so in the winter months when the temperature is low. This problem was decreased or avoided by heating the blank wax blocks slightly before starting the procedure. The slight heating makes the wax more moldable and hence prevents breakage.

If the donor core tissue is longer than the hole punched out, the tissue core will not be completely in line with the surface of the block. If the core is smaller, it will again leave some empty space in the block and the block may collapse.

30 cases of breast cancer were studied and the largest number of patients were in the 31-50 years age group comprising 20/30 cases. This was followed by a decrease in the number of cases in the higher age groups. Althuis et al [8] also stated that a steep rate of increase was evident among pre-menopausal women residing in Asia and the post-menopausal rates plateau and even decreased in some countries. Another study [9] also found that in India incidence appears to decrease with age beginning at age 50.

20% cases (6/30) in this study were found to be HER-2 protein positive. While some studies found HER-2 expression in 10 – 20% of the cases of breast cancer [10], other studies reported a wider range from 10 to 34% [11]

The HER-2 expression was compared with the other morphological parameters including grade, tumour size, lymph node status, presence of in situ component and lymphovascular invasion. A higher percentage of HER-2 expression was found in higher grade tumours and tumours with a larger size. Neither of this was found to be statistically significant. HER-2 expression was also found to be higher in high N stage group. This was statistically significant with a P value of <0.05.

Berger et al [11] did a study in which they found that HER-2 expression correlated with tumour grade and lymph node status in an univariate analysis. Wright et al [12] in their study also found a correlation of HER-2 expression with tumour grade in both univariate and multivariate analysis. They however did not find any correlation with tumour size and lymph node status.

Noguchi et al, Tiwari et al, Descotes et al and Hartmann et al reported a correlation with higher lymph node status and HER-2 expression in univariate analysis. Lee a et al found over expression of HER-2 to be associated with a larger tumour size and lymph node metastasis.

In our study, statistically significant result was obtained for HER-2 protein expression with higher nodal involvement. The HER-2 expression was increased with the lymphovascular invasion, which is a surrogate morphological marker for lymph node involvement by the disease.

7/30 cases (23%) were found to be positive for HER-2 gene amplification by CISH. The number of cases with HER-2 gene over expression also increased from low stage to higher stage group (AJCC). The highest number of positive cases were seen in PT4 stage (3/7) and (2/7) cases each in N2 and N3 stage. Tumours with grade 3 showed maximum positivity of (3/7) positive cases.

The main purpose of our study was to use manually constructed tissue microarrays for the detection HER-2 by CISH and IHC in breast cancer and to study the degree of agreement between the results obtained on CISH (which was taken as the gold standard) with the IHC tissue microarray sections. This was done in an attempt to validate the use of tissue microarray sections routinely in the future for research and diagnostic purpose in resource limited laboratories

The Cohens kappa value that was calculated was 0.510 between HER-2 protein and HER gene which indicates moderate agreement between them.

A perfect agreement with a Cohens kappa between 0.81 to 1 was however not found between HER-2 IHC and HER-2 gene by CISH. This can be attributed to many factors. A small core of the entire tumour tissue is subjected to staining in a tissue microarray. The IHC staining pattern of that small core may not be an ideal representation of the entire tissue. Also, the tumour may itself be morphologically heterogeneous.

2/6 HER-2 protein positive cases were negative for HER-2 gene. This discrepancy could be due antibody recognizing other proteins which are members of EGFR family or due to degradation of nucleic acid by bad fixation. 3/6 cases positive for HER-2 gene were negative for HER-2 protein. This could be due to polysomy or pseudogenes being hybridized by probes. Laia et al studied 403 cases for HER-2 protein and HER-2 gene. They found 108 discordant cases between HER-2 protein and gene amplification. Out of 108 cases, 20 were protein positive/gene negative and 88 were protein negative/gene positive.

Another common problem faced in using the tissue microarray section is the loss of core. This can be reduced to a significant extent by the use of poly-lysinated slides for IHC.

The loss of core during taking sections can be significantly reduced by properly cooling the block on ice and using fresh blades. The TMA blocks should not be subjected to much trimming .

The use of tissue microarray sections significantly decrease the amount of time and reagents used for IHC. Since we used a 3X5 grid, for 30 cases, two tissue microarray sections were processed. CISH reagents being very costly and since our kits had only capacity for performing ten slides, without TMA sections and slides it might have not been possible to do 30 cases in our study. As the entire cohort of cases being stained together and subjected to the exactly same processing, variations in staining due to changes in the pH, temperature, staining time etc were not seen. This study, hence validates the use of tissue microarray for IHC and CISH analysis of breast cancers for HER-2 over expression for research and diagnostic purposes.

Conclusion

1. HER-2 expression in carcinoma breast could be studied by IHC and CISH with ease on Tissue microarray sections.
2. This Study found moderate agreement between HER-2 protein by IHC and HER-2 gene by CISH.
3. The low cost of manually creating such microarrays along with the decreased requirement of the reagents was found to be highly cost-effective and resource saving in IHC and CISH.
4. CISH does not require fluorescence microscopy like FISH and nuclear signals does not fade away.
5. The use of tissue microarray sections for CISH and IHC substantially reduced the total time required for staining.

Recomendations

1. The use of more than a single core from each case will further improve the agreement.
2. Given all the inherent advantages of the tissue microarray technology, this technique can be further standardized for use in routine and research purposes in developing countries like India.

Reference

1. Darryl C, Stuart J, Rosemary R. The breast. In: Diagnostic surgical pathology. 5th edition. New York: Wolters Kluwer; 2010;1:285.
2. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177-82.
3. Yeole BB, Kurkure AP. An epidemiological assessment of increasing incidence and trends in breast cancer in Mumbai and other cities in India, during the last two decades. Asian Pacific Journal of Cancer Prevention. 2003;4:51-56.
4. Kumamoto H, Sasano H, Taniguchi T, Suzuki T, Moriya T, Ichinohasama R. Chromogenic in situ hybridization analysis of HER-2/neu status in breast carcinoma: application in screening of patients for trastuzumab (Herceptin) therapy. Pathol Int. 2001;51:579-84.
5. Singh DK, Sakhuja P, Gondal R. Making and Using inexpensive manually constructed tissue micro-array: Experience of a tertiary care hospital in India. Indian Journal Of Pathology and Microbiology. 2009;52(3): 304-309.
6. Onitilo A, Engel JM, Greenlee RT, Bickol NM. Breast Cancer Subtypes Based on ER/PR and Her2 Expression: Comparison of Clinicopathologic Features and Survival. Clinical medicine and research. 2009;7(1):4-13.
7. Indrayen A. Relationships: qualitative dependent. In: Indrayen A, editor. Medical Biostatistics. 2nd edition. New-York: Chapman and Hall/CRC; 2008:571-599.
8. Althuis MD, Dozier JM, Anderson WF, Devesa SS, Brinton LA. Global trends in breast cancer incidence and mortality 1973-1997. International Journal of Epidemiology 2005;34:405-412.
9. Okonkwo QL ,Draisma G, der Kinderen A, Brown ML , de Koning HJ. Breast Cancer Screening Policies in Developing Countries: A Cost-effectiveness Analysis for India. Journal of the National Cancer Institute. 2008;100:1290–1300
10. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, Slamon DJ, Murphy M, Novotny WF, Burchmore M, Shak S, Stewart SJ, Press M. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2- overexpressing metastatic breast cancer. J Clin Oncol. 2002;20:719-26.
11. Berger MS, Locher GW, Saurer S et al. Correlation of c-erb B2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Research. 1988;48:1238-1243.
12. Wright C, Angus B, Nicholson S et al. Expression of c-erb B-2 oncoprotein: a prognostic indicator in human breast cancer. Cancer Research. 1989;49:2087-2090.