

Surrogate molecular subtyping of breast carcinomas – A study on recent modifications and their clinicopathological significance

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Context: Breast carcinoma is a heterogenous disease with varied clinicopathological features and response to therapy. Molecular classification through gene studies helps in planning therapy but has economic constraints. Hence immunohistochemical subtyping of breast carcinomas has been used as a surrogate method. Criteria for this subtyping has undergone many modifications since it was originally proposed. **Objectives:** To immunohistochemically subtype breast carcinomas based on St.Gallen 2017 guidelines and analyse the differences in clinicopathological parameters like age, tumour size, histopathological grade and lymph node staging between the various subtypes. **Materials and methods:** The study was done retrospectively at a tertiary care health centre in South India on breast carcinoma patients from January 2017 to June 2020. Immunohistochemistry was done with antibodies to the Estrogen receptor, Progesterone receptor, Human epidermal growth factor receptor-2 (HER-2) and Ki-67. Immunohistochemical Subtypes were correlated with Clinicopathological features. **Results:** The study had 107 cases. Hormone receptor (HR) positive HER-2 negative was the most common subtype (55 cases, 51.4%). This subtype frequently presented without nodal metastasis (58.2%) and in >50 years of age (56.4%). Triple-negative subtype frequently presented with grade III (69.2%), highest nodal metastasis stage (38.5%) and in < 50 years of age (69.2%). **Conclusion:** St.Gallen 2017 guidelines for immunohistochemical subtyping classified breast carcinomas into groups that differed significantly in their clinicopathological features. Further studies on differences in treatment response and survival rate differences between these different subtypes are needed.

Keywords: Breast carcinoma, Immunohistochemistry, Molecular subtyping, St. Gallen

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Introduction

Breast cancer is one of the leading causes of cancer-related mortality and morbidity in females. Various advancements have been made in the diagnosis and treatment of breast cancer in recent decades. Due to these advancements in breast cancer therapy, the current focus is on identifying the optimal treatment strategy for individual breast cancer patients. For this purpose, breast cancer patients can be classified into various subgroups based on their clinicopathological features and the ideal treatment for each subgroup is being studied [1]. These clinicopathological variables, otherwise called the “predictive markers” indicate the sensitivity or resistance to various forms of therapies in a particular patient thereby helping to choose the ideal therapy for them.

The molecular subtype is one such predictive marker that has got prognostic significance as well [2]. There are 5 molecular subtypes of breast cancer: Luminal A, Luminal B, HER-2-neu enriched, Basal-like and Normal breast-like. Although these subtypes indicate the response to various forms of therapies, identifying the molecular subtype in each patient needs gene assays which are costly and need advanced equipment as well. Such facilities might not be available routinely across all the centres, particularly in the developing and under-developed countries.

Hence surrogate subtyping of breast cancer has been proposed based on immunohistochemical features which give results closer to the molecular subtyping. However, the criteria for this surrogate subtyping has constantly undergone modifications based on various studies. The recent 15th St. Gallen International Expert Consensus Conference on Breast Cancer held in the year 2017 has further modified the criteria for this surrogate subtyping [1]. Hence this study was done to assess the frequency of different intrinsic subtypes of breast cancer and their clinicopathological features based on these recent St. Gallen’s modifications.

Materials and methods

Place of study: Our study was done at a tertiary care health centre in South India from April 2019 to June 2020.

Inclusion criteria: All cases of invasive breast carcinomas in which modified radical mastectomy had been done and received in the Department of

Pathology from January 2017 to June 2020 were included in our study.

Exclusion criteria: Post-chemotherapy patients and cases with insufficient material in blocks for immunohistochemical staining were excluded from the study. This study was approved by the Institutional Ethics Committee.

Interpretation criteria: In all these cases, specimens had been received in 10% formalin and according to the standard surgical grossing protocol appropriate bits were given. Haematoxylin and Eosin (H&E) stained slides were examined to confirm the histological diagnosis. Histological grading was performed by Nottingham’s modification of the Bloom-Richardson grading system.

Following the 8th edition of the American Joint Committee on Cancer (AJCC) cancer staging guidelines tumour size in the greatest dimension was considered. Tumours up to 20mm were included under T1, >20mm up to 50mm under T2 and >50mm under T3. Tumour of any size with direct extension to the chest wall and /or to the skin was included in T4. If no regional lymph node metastasis could be detected they were categorized as N0, metastasis in 1-3 axillary lymph nodes as N1, metastasis in 4-9 axillary lymph nodes as N2 and metastasis in >10 axillary lymph nodes as N3.

For the immunohistochemistry (IHC) procedure, the peroxidase anti peroxidase (PAP) method was followed. Representative blocks were submitted for IHC after screening all sections. 3-5 micron thickness sections were taken on positively charged slides and incubated. Slides were deparaffinised and dehydrated in xylene, absolute alcohol. The antigen retrieval process was done in microwave cooking with Tris-EDTA (ethylenediaminetetraacetic acid) buffer solution maintained under pH 9.

Then sections were treated with 3% hydrogen peroxide. Sections were stained with primary antibody for 1 hour. Antibodies to estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor- 2 (HER-2) were used. All were monoclonal rabbit antibodies (PathnSitu). Then polyexcel horseradish peroxidase (HRP) labelled secondary antibody was added and incubated. Then 3, 3’diaminobenzidine (DAB) chromogen was added and counterstained with Harris haematoxylin for 30 seconds.

As per the American Society of Clinical Oncology/

College of American Pathologists (ASCO/CAP) guidelines ER/PR were considered positive when 1% or more cells showed nuclear staining. For HER -2, if there were no membranous staining or faint incomplete membranous staining in less than 10% of cells, the score was given as zero.

Faint incomplete membranous staining in >10 % of cells was scored 1+. Weak to moderate complete membranous staining in >10% cells was scored 2+. Strong complete membranous staining in >10% cells was given a score of 3+. Cases with score 0 and 1 were considered HER-2 negative. Score 3+ was taken as HER-2 positive. Score 2 was taken as an equivocal result and Fluorescence in situ hybridization (FISH) was done in these cases to confirm HER-2 status.

Based on the expression of ER, PR and HER-2, all the cases were subtyped into four categories according to St. Gallen 2017 guidelines. These four categories were triple-negative (hormone receptor (HR) negative, HER-2 negative) tumours, HR negative HER-2-positive tumours, HR-positive HER-2 positive tumours and HR-positive HER-2 negative tumours. The last group was further classified into "Luminal A-like" (low grade) and "Luminal B-like" (high grade) tumours.

For grade II cases, Ki-67 immunohistochemical staining (PathnSitu) was done and when <20% cells were positive they were categorised as "Luminal Alike" tumours. When >20% cells were Ki-67 positive they were categorised as "Luminal B- like tumours". Clinicopathological parameters like age, T (tumour size) staging, N (lymph node) staging and histopathological grading were studied in all these groups and compared.

Statistical analysis: Statistical analysis of the data was done using SPSS software 24.0 by Chi-square test. p-value less than 0.05 was considered statistically significant.

Results

Our study had 107 cases based on the inclusion and exclusion criteria. HR-positive HER-2 negative subtype (55 cases, 51.4%) was the most common subtype in our study with 38 "luminal Alike" cases (35.5%) and 17 "luminal B- like" cases (15.9%). This was followed by HR negative HER-2 positive and triple-negative subtypes (Table I). HR-positive HER-2 positive subtype was the least common subtype in our study with 11 cases (10.3%). These subtypes showed significant differences in their clinicopathological features.

Triple-negative subtype cases more often presented with grade III (69.2%). HR-positive HER-2 positive (81.8%) cases more often presented with grade II. Triple-negative, HR-positive HER-2 positive subtypes never presented with grade I (Table II). On the other hand Grade I tumours were more common in HR-positive HER-2 negative subtype when compared with other subtypes. The association between various subtypes and grade was found to be statistically significant (p-value <0.001).

Triple-negative subtype cases also frequently had the highest stage of lymph node metastasis (N3-38.4%), whereas HR-positive HER-2 negative subtype often did not show nodal metastasis (N0-58.2%)(Table III). In the latter subtype, "luminal Alike" cases often did not show nodal metastasis (76.3%) compared to "luminal B -like cases (17.6%) The association between various subtypes and lymph node staging was found to be statistically significant (p-value <0.001).

TABLE I: Immunohistochemical subtypes of breast carcinoma in our study

Immunohistochemical subtypes	Number of cases (%) ; n=107
Triple negative	13(12.1%)
HR -,HER-2 +	28(26.2%)
HR+, HER-2 +	11(10.3%)
HR+,HER-2 -	55(51.4%)

(HR- hormone receptor; HER-2 -human epidermal growth factor receptor -2)

TABLE II: Histological grade in different immunohistochemical subtypes of breast carcinoma

Immunohistochemical subtypes	Grade I	Grade II	Grade III	χ ² -Value	p-Value
Triple Negative (n=13)	0(0.0%)	4(30.8%)	9(69.2%)	27.087	<0.001
HR-,HER-2+ (n=28)	2(7.1%)	19(67.9%)	7(25%)		
HR+,HER-2+ (n=11)	0(0.0%)	9(81.8%)	2(18.2%)		
HR+,HER-2- (n=55)	15(27.3%)	36(65.4%)	4(7.3%)		

(HR- hormone receptor; HER-2 -human epidermal growth factor receptor -2)

TABLE III: Lymph node staging in different immunohistochemical subtypes (N stage)

Immunohistochemical subtypes	N0	N1	N2	N3	χ ² -Value	p-Value
Triple Negative (n=13)	2(15.4%)	2(15.4%)	4(30.8%)	5(38.4%)	54.528	<0.001
HR- , HER-2+ (n=28)	4(14.3%)	5(17.8%)	19(67.9%)	0(0.0%)		
HR+, HER-2+ (n=11)	4(36.4%)	5(45.4%)	0(0.0%)	2(18.2%)		
HR+, HER-2- (n=55)	32(58.2%)	17(30.9%)	4(7.3%)	2(3.6%)		

(HR- hormone receptor; HER-2 –human epidermal growth factor receptor -2)

TABLE IV: Tumour staging in different immunohistochemical subtypes (T stage)

Immunohistochemical subtypes	T1	T2	T3	T4	χ ² -Value	p-Value
Triple Negative(n=13)	0(0.0%)	8(61.5%)	4(30.8%)	1(7.7%)	31.395	<0.001
HR- ,HER-2+ (n=28)	0(0.0%)	20(71.4%)	8(28.6%)	0(0.0%)		
HR+,HER-2+ (n=11)	2(18.2%)	8(72.7%)	0(0.0%)	1(9.1%)		
HR+,HER-2- (n=55)	16(29.1%)	37(67.3%)	2(3.6%)	0(0.0%)		

(HR- hormone receptor; HER-2 –human epidermal growth factor receptor -2)

TABLE V: Immunohistochemical subtypes and Age

Immunohistochemical subtypes	Below 50 years	Above 50 years	χ ² -Value	p-Value
Triple Negative (n=13)	9(69.2%)	4(30.8%)	3.001	0.401
HR- ,HER-2+ (n=28)	12(42.9%)	16(57.1%)		
HR+,HER-2+ (n=11)	5(45.5%)	6(54.5%)		
HR+,HER-2- (n=55)	24(43.6%)	31(56.4%)		

(HR- hormone receptor; HER-2 –human epidermal growth factor receptor -2)

Percentage of larger tumours (T3) was maximum in the triple-negative subtype (30.8%) and HR negative HER-2 positive subtype (28.6%) when compared to other subtypes (Table IV). On the other hand, smaller tumours (T1) were more frequent in the HR-positive HER-2 negative group (29.1%).

In this subtype also smaller tumours were more common in “luminal Alike” cases (42.1%) compared to “luminal B- like” cases. Triple-negative and HR negative HER -2 positive rarely presented with T1 tumours. The association between various subtypes and tumour size was also found to be statistically significant (p-value < 0.001).

With regards to age, triple-negative (69.2%) subtype often presented in women of the age group of fewer than 50 years (Table V). All the other subtypes were more common in women of more than 50 years. On the statistical analysis of data, the association between various subtypes and age was not significant (p value=0.401).

Discussion

Breast cancer is a heterogeneous disease with varied clinicopathological features. As a result all the cases do not respond uniformly to the different forms of therapy.

Molecular classification of breast carcinomas through gene profiling studies is a useful tool in planning therapy for breast cancer patients as some subtypes respond well to endocrine therapy whereas others respond better to chemotherapy. But due to the lack of facilities and increased expenditure it cannot be used in day to day practice in all economic setups. Therefore immunohistochemistry based subtyping of breast carcinomas was suggested as a surrogate method and is being widely used [3]. This subtyping of breast cancer based on immunohistochemistry has been constantly under evolution with repeated modifications in criteria based on new studies, advancements and practical experience. The St. Gallen's Guidelines for immunohistochemical subtyping of breast carcinomas which has been accepted by the World Health Organization was first suggested in 2011 [4] and has undergone various modifications in the subsequent St.Gallen conferences. This study was based on the recent 2017 guidelines [1] which had recommended that molecular subtype terms should not be used in the surrogate immunohistochemical subtyping as the results differ in some cases. However the guidelines recommended that immunohistochemical subtyping of breast carcinomas should be continued but with certain modifications in the types, terminology and criteria.

They further recommended this classification to be used in planning treatment strategy in individual breast carcinoma patients. HR-positive HER-2 negative was the most common subtype in our study. This in turn was due to the high frequency of "luminal Alike" cases. In the earlier classification, "luminal A" used to be a separate subtype and was the most common subtype in many previous studies [3,5,6,7,8,9]. Studies on the Indian population done by *Munjal et al* [10] and *Ashima et al* [9] had also shown "luminal Alike" as the predominant subtype. However few other Indian studies by *Shabnam et al* [11] and *Kunheri et al* [12] observed triple-negative and "luminal B-like" to be the most common subtypes respectively. It needs to be studied further whether this is a geographic variation in different parts of India due to some genetic and geographic factors.

Subtypes and tumour size: Our study found a significant association between the subtypes and the tumour size. The proportion of tumours larger than 5cms was maximum in triple-negative subtype in our study. *Spitale et al* [3] had observed the same in their study. After the triple-negative subtype, the frequency of such larger tumours was maximum in the HR negative HER-2 positive subtype. Studies by *Onitillo et al* [13] and *Engstrom et al* [14] had also observed that tumours larger than 5cm were frequent in this subtype. At the other end, tumours less than 2cms were frequent in the HR-positive HER-2 negative subtype particularly in the "Luminal Alike" cases in our study. Such an association between small tumour size and "Luminal Alike" cases was noted in many previous studies as well [3, 5, 7, 13, 14]. Although few studies have noted no significant association between subtypes and tumour size they seem infrequent [10,15].

Subtypes and lymph node metastasis: The triple-negative type was frequently associated with the highest stage of lymph node metastasis in our study. In contrast to our results nodal metastasis was frequently absent in triple-negative subtype in studies by *Spitale et al* and *Vasconcelos et al* [3, 5]. However many other study results by *Zaha et al*, *Munjal et al*, *Kunheri et al* and *Blows et al* were similar to our study strengthening our results [6,10,12,16]. Lymph node metastasis was often absent in "Luminal Alike" tumours in our study. This was similar to the results of *Tang et al* [17] and *Onitillo et al* [13].

Subtypes and tumour grade: Triple-negative subtype cases were often of the highest histological

Grade in our study similar to previous studies [3, 5,7,12,15]. "Luminal Alike" cases showed the maximum percentage of Grade I tumours. These findings were also consistent with previous [3,5,8,10,13,14,15] studies. Statistically significant difference was noted in the histological grade between various subtypes in our study, similar to many previous studies [3,6,7,10,15,16,18,19].

Subtypes and age of the patient: Although our data showed no significant difference in the age of presentation in various subtypes (p-value = 0.401), our results were comparable to previous studies. Triple-negative subtype involved a younger age group in our study, similar to studies by *Spitale et al*, *Vasconcelos et al* and *Yinghao et al* [3,5,8]. "Luminal Alike" cases presented in an older age group in our study, a finding that was noted in *Spitale et al* [3] and *Yinghao et al* [8] studies as well. Thus although statistical significance was not reached, our results were in line with the previous studies.

New entities in recent classification: HR-positive HER-2 positive and HR-positive HER-2 negative were two new entities proposed only in the latest St. Gallen guidelines. Hence we could not compare our results in these groups with many previous studies. HR-positive HER-2 positive subtype frequently presented in more than 50 years women with T2 size and grade II tumours. HR-positive HER-2 negative subtype also frequently presented with similar features but with less frequent nodal metastasis. Although some of these findings were similar to the study by *Cheng et al* [20], we could not compare our results with most studies since these two are new entities that came only after the new classification. Being new entities, more studies need to be done which might confirm our finding.

Significance of "luminal Alike" and "luminal B-like" tumours: One of the major grey zones in the immunohistochemical subtyping of breast carcinomas is in distinguishing the "Luminal A-like" and "Luminal B-like" tumours among the HR-positive HER-2 negative cases. In fact this was the major area of a dilemma for so many years and so many modifications were repeatedly being done in the criteria to distinguish these two entities. When we classified these tumours based on the recent St. Gallen guidelines [1], cases in the resulting two groups differed significantly in their clinicopathological features reiterating the validity of these guidelines.

While “Luminal A- like” tumours were often of smaller tumour size without nodal metastasis in many cases, “Luminal-B” like tumours were often of a larger tumours size with nodal metastasis.

One of the limitations of our study was we were not able to do a long term follow up of these patients to assess the difference in treatment response and survival rates among the different subtypes. This was because many patients were lost to follow-up. Another limitation was Ki-67 was not done in all cases due to economic constraints.

However this was not necessary in all cases as according to the St. Gallen guidelines, the grade of the tumour can be used instead of Ki-67 for classifying the “Luminal A- like” and “Luminal B-like” tumours. We followed the same guidelines. In cases where the grade was intermediate alone, we used Ki-67 for classification. Such an approach would be cost-effective for subtyping breast carcinomas in developing countries.

Conclusion

Breast cancer is a heterogeneous disease with different subtypes differing in their clinicopathological features. Since these subtypes are likely to differ in their treatment response as well, it is essential to identify the subtype in every individual patient.

Since molecular subtyping might not be affordable in developing and undeveloped countries, surrogate immunohistochemical subtyping can be used. We adopted the recent St. Gallen guidelines for this immunohistochemical subtyping and observed that the subtypes differed significantly in their clinicopathological features further validating the guidelines.

Further long term studies with a large sample size and adequate follow up will help in assessing the treatment response and the survival rates in these different subtypes. Such large scale studies will help in planning the treatment guidelines for each subtype particularly in the Indian population.

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