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Original Article

DOI: 10.53389/RJAHS.2024030203

Molecular Subtypes of Breast Cancer and Their Clinicopathological Correlations in a Large Cohort from Pakistan Using Immunohistochemistry

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Author`s Contribution

[AA]Conception and design, [SA, GM] Collection and assembly of data, [GM]Analysis and interpretation of the data, Statistical expertise, [MA, AA] Final approval and guarantor of the article

Article Info.

Received: Nov 12, 2024
Acceptance: Dec 05, 2024

Conflict of Interest: None
Funding Sources: None

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A B S T R A C T

Background: Breast cancer is a heterogeneous disease with many different molecular subtypes that affect prognosis and response to treatment. Immunohistochemical profiling for estrogen receptor, progesterone receptor, and HER2/neu provides a convenient method of subclassifying breast tumors and directing clinical management. Understanding the distribution and clinicopathological correlations associated with these subtypes can guide improvements in therapeutic strategies.

Objective: The present study is aimed to evaluate the frequency of molecular subtypes of breast cancer by immunohistochemistry and their relation with various clinicopathological features among the patients in Punjab, Pakistan.

Methodology: A cross-sectional study was conducted from 2023 to 2024 at Histopathology Laboratory of Fatima Memorial Hospital and the Department of Microbiology, University of Central Punjab, Lahore, using formalin-fixed, paraffin-embedded (FFPE) tissue specimens from 800 patients with a confirmed diagnosis of invasive ductal carcinoma of the breast. All cases underwent immunohistochemical staining for estrogen receptor, progesterone receptor, and HER2/neu for classification into molecular subtypes. Clinicopathological data including age, tumor grade, menopausal status, tumor size, lymph node involvement, and family history were noted. Statistical analysis was done using descriptive statistics and chi-square tests.

Results: Among the patients, luminal A was the most frequent molecular subtype (30.6%), followed by luminal B (29.3%), triple negative (23.1%), and HER2-enriched (17.0%). Moderately differentiated tumors accounted for 55.4% of cases. Premenopausal women predominated in luminal A and B subtypes, whereas triple negative tumors were more common in postmenopausal women. The HER2-enriched subtype showed the highest rate of lymph node involvement (69%). Tumor size was largest in luminal A cases. Family history was most frequently observed in the luminal A subtype. These associations were statistically significant.

Conclusion: The outcome of this investigation reveals the relative incidences and clinicopathological correlations of breast cancer molecular subtypes in a defined population. Immunohistochemistry is crucial for individualized treatment planning, which in turn will enhance clinical outcomes.

Keywords: Breast Neoplasms, Molecular Subtyping, Immunohistochemistry, Estrogen Receptor, Progesterone Receptor, HER2 Protein.

Introduction

Breast cancer is a broad term that encompasses a variety of conditions with different clinical and cytopathological characteristics, and outcomes. However, this is no longer the case because breast cancer with indistinguishable histopathological and clinical characteristics might have varied outcomes and chemotherapy susceptibility.[1] Breast cancer has been found to exhibit a range of molecular genetic changes, which are diverse and complicated in nature. Because of the

molecular and cellular diversity of the breast tumor, as the increased group of genes that can influence growth, death, and cell proliferation, several genes' mutations are examined simultaneously.[2] Breast cancer appears to be a collection of multiple disorders with distinct risk variables, clinical signs, histological characteristics, responsiveness to treatments, and prognosis that impact similar body tissue and begin in the same terminal duct lobular region, according to numerous bits of research.[3] Even though researchers have long been recognizing the diversification, work to develop a method for classifying it into predefined categories has only been recently brought to the light of breast tumor biomedical research following the release of elevated-throughput microarray-based category advancement analyses that disclosed the presence of various molecular subtypes.[4]

As per tumor grade and histopathological nature, breast tumors can be divided into medically and physically relevant subtypes. The extent of changes in the form of pathologically elevated tube production, nuclear differentiation, and cellular reproduction index also known as the mitotic activity of tumors is represented in its grade, which reflects its severity. Numerous established prospective systems, such as the Nottingham histological scoring system, utilize histological tumor grade to select breast cancer treatment.[5] Surprisingly, grading is linked to the genomic and transcriptional characteristics of breast tumors, and microarray-dependent genomic markers indicating histopathological molecular subtype have been established. Contrary to this, the histological subtype describes the characteristic of the cancer growth pattern.[6] Pathologists have long been captivated by the histopathological heterogeneity of breast carcinomas, and have found certain structural and cytogenic features that are directly related to variable clinical appearance and consequences. 'Molecular histological subtypes' are the terms used to refer to these patterns. Invasive ductal breast carcinoma of no special type is known to be the most frequent kind of breast cancer — this is an exclusionary classification that incorporates carcinomas that don't show specific clinical features and do not meet the criteria for categorization in a few of the special types. The foundation of an evolved terminology for breast cancer is the comprehensive evolution of genetic patterns and their correlation with different features of phenotype variability in tumors employing comparable genetic profiling methods. These investigations have identified several biological groups of breast cancer, such as HER-2.neu overexpression, luminal B, triple-negative, and luminal A. Chemotherapy forecasts and consequences differ significantly by subtype.[7] Molecular therapy is credited with being the first to treat this dreadful disease. Molecular categorization of breast tumors is more expensive and difficult to get by. Immunohistochemistry identifies a wide range of target genes and can be used instead of genetic analysis. This is closely related to genetic analysis and can even be considered too as low-cost genetic testing. The immunohistochemistry (IHC) categorization can help with both treatment and prognosis.

Breast cancer will be classified into four categories in the study relying on IHC patterns of estrogen receptor, progesterone receptor, as well as HER 2 neu activity. Certain breast cancer cells require estrogen and progesterone (naturally occurring hormones) to proliferate. Hormone receptors are unique proteins found inside the tumor cell. Cancer cells with hormone receptors thrive when hormones bind to these receptors. Hormonal therapy is active against breast cancers that have hormone-positive receptors and HER2 neu-positive cancers indicate susceptibility to monoclonal antibody-based target therapy. Amidst having a generally better survival, some individuals with the luminal breast tumor subclass experience lethal resurgence while still being on hormone therapy. At least two subgroups of luminal breast tumors — called luminal A and B — have now been detected by gene expression research investigations, each with a varied outlook response. Even though this categorizing approach has been proved to be predictive and therapeutically useful, it does not properly represent the diversity of luminal tumor.[8]

The size of the cancer (T), dissemination to lymph nodes (N), and metastasis status (M) during the detection have a significant impact on the outcome of invasive ductal carcinomas of the breast.[9] Furthermore, commonly performed IHC immunoreceptors, as well as grade, are highly predictive of breast cancer outcomes and advised treatment recommendations in the past several decades in conjunction with TNM.[10] Even though validated IHC receptors, grade, and TNM have unquestionable diagnostic significance, they are rarely examined extensively, even in large settings. With the emergence of new therapeutics, it is critical to constantly re-determine the function of traditional diagnostic factors in big community statistics to verify and, if required, revise cancer categorization. These updated findings will aid biological researchers in their search for novel indicators for clinical populations with insufficient predictive descriptions.

The present study aims to determine the distribution of molecular subtypes of breast cancer through immunohistochemical analysis, evaluate their correlations with key clinicopathological features, investigate the association between molecular subtypes and histological tumor grade, and identify the predominant breast cancer subtype within the population of Punjab, Pakistan. These objectives seek to

provide insights that can facilitate the development of more targeted and effective therapeutic strategies for breast cancer management in this region.

Materials and Methods

The period of study extends from 2023 to 2024, with data collection occurring at the Histopathology Laboratory of Fatima Memorial Hospital and the Department of Microbiology, University of Central Punjab, Lahore. Ethical approval from the institutional review board was deemed necessary prior to the commencement of the study. A total of 800 formalin-fixed paraffin-embedded (FFPE) breast tissue blocks diagnosed as invasive ductal carcinoma were selected as the study population. Tissues were selected according to a non-probability purposive sampling method. Tissue with a confirmed histopathological diagnosis and with sufficient tissue were included while excluded were specimens that were poorly preserved or had improper documentation.

The tissue sections of 2 microns were cut through a Leica RM2125-RTS microtome and floated in a water bath kept at a temperature of 45°C before mounting on Dako-coated super frosted slides. These were then air-dried for rigorous adherence. Deparaffinization was done on a 90°C hot plate, followed by immersion in xylene and in three changes of isopropanol, with subsequent rehydration in distilled water. Antigen retrieval was performed using diluted Envision FLEX EDTA/Tris buffer (pH 9) at 99.9°C for 45 minutes in a water bath; cooling of the slides was subsequently done.

Dako FLEX peroxidase blocking reagent anti-endogenous peroxidase. After application of 50 µl each primary antibodies: estrogen receptor (ER), progesterone receptor (PR) and HER2/neu onto slides, the slides were then allowed to incubate in a humidity box at room temperature for one hour. After some washing, the slide was again incubated for the next hour with horseradish peroxidase conjugated secondary antibody. Antigen-antibody reaction was visualized by using diaminobenzidine (DAB) whose end product would form a brown precipitate at the site of positive staining. The slides were counterstained with Harris's hematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted with DPX and borosilicate coverslips.

Two well-qualified pathologists independently assessed the stained slides using an Olympus compound microscope. The scoring of estrogen receptor (ER) and progesterone receptor (PR) expression was based on the Allred scoring system, which considers both the percentage and intensity of cell staining. A score of 0 was assigned to indicate the absence of staining; a score of 1 indicated staining of fewer than 1 in 100 cells weakly (week positive); a score of 2 was given to staining in 1 in 100 to 1 in 10 cells with intermediate intensity (intermediate positive); while a score of 3 indicated staining in 1 in 10 to 1 in 3 cells with strong intensity (strongly positive).

HER2/neu expression was determined according to Envision system criteria, which evaluate both intensity of staining and percentage of membranous staining. A score of 0 was assigned for none of the immunohistochemically (IHC) stained cells; A score of 1 was assigned for less than 30 % weakly stained cells; A score of 2 was given when 10 % of the cells showed intermediate staining; A score of 3 designated 30 % or more of the cells showing strong membranous staining.

Interpretation of IHC results followed ASCO/CAP guidelines. Data were entered and analyzed using SPSS version 26. Categorical variables were expressed as frequency and percentage. Chi-square test was used to determine the association between IHC-based molecular subtypes and clinicopathological variables. A p-value of less than 0.05 was considered statistically significant. Due to the retrospective nature of tissue availability, no formal sample size calculation was done, although a sample of 800 was considered sufficient based on similar studies to allow robust statistical analysis.

Results

The examination comprised 800 breast cancer tissue samples diagnosed as invasive ductal carcinoma. The patients' ages ranged from 11 to 90 years. The age group with the highest case frequency was 41–50 years, which represented 29.30% of the study population, while the ranges of 31–40 years and 51–60 years represented 25.60% and 23.50% of the cases, respectively. The least represented group was the 11–20 years category, with 0.30% of cases (Table I).

Table I: Distribution of age ranges of patients.

Patient Age Range	Frequency	%
11-20	2	3
21-30	42	5.3
31-40	205	25.6
41-50	234	29.3
51-60	188	23.5
61-70	92	11.5
71-80	28	3.5
81-90	9	1.1
Total	800	100.0

Histopathological evaluation entailed scoring three principal parameters: glandular/tubular differentiation, nuclear pleomorphism, and mitotic count. The majority of tumors (77.60%) showed a score of 3 for glandular differentiation, indicating poor formation of glands in most of the cases.

DOI: 10.53389/RJAHS.2024030203

Nuclear pleomorphism was most frequently given a score of 2 in 53.10% of cases, while mitotic activity was mostly given a score of 2 in an even greater frequency of 54.40% of samples. Rarely did each parameter have a lower score (1) (Table II).

Table II: Frequency of Scoring Levels of Pathological Features. (n = 800)

Score	Glandular/Tubular Differentiation	Nuclear Pleomorphism	Mitotic Rate
1	20 (2.50%)	102 (12.80%)	200 (25.00%)
2	178 (22.30%)	425 (53.10%)	435 (54.40%)
3	621 (77.60%)	355 (44.40%)	165 (20.60%)

Scoring in the Nottingham Histological Grading System combines the three types of individual scores mentioned before. A score of 6, which refers to intermediate-grade tumors, was most frequent in 43.60% of cases, with 32.40% scoring 8. High-grade tumors in this study scored a 9 in 9.10% of cases, while score 4, the lowest combined score in this study, was recorded in only 0.30% of cases (Table III).

Table III: Frequency of Nottingham Histological Scores. (n = 800)

Score	Frequency	(%)
4	2	0.30
5	29	3.60
6	349	43.60
7	88	11.00
8	259	32.40
9	73	9.10

In their histological grading, most of the tumors were moderately differentiated at grade II, which contributed to a significant 55.40% out of the total cases. Poorly differentiated tumors at grade III comprised 41.50% while those well-differentiated at grade I represented the lowest number, with only 3.10% (Figure 1).

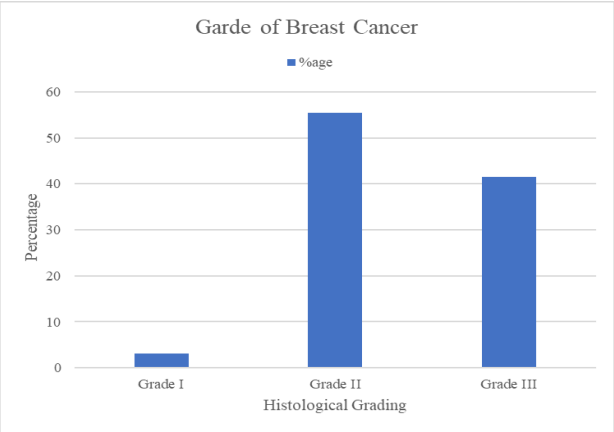

Figure 1. Frequency of Histological Grades.

The breast cancer receptors panel which included estrogen receptor, progesterone receptor, and HER 2 neu was performed on these 800 samples of breast cancer using immunohistochemistry to analyze the staining characteristics & pattern of breast tumors which are described (Figure 2). Out of 800 samples, 475 (59.4%) showed positive reactions, and 325 (40.6%) showed negative reactions for estrogen receptors. Progesterone receptors were positive in 393 (49.1%) samples and negative in 407 (50.9%). While Her 2 neu had given positive staining in 361 (45.1%) samples and negative staining in 439 (54.9%).

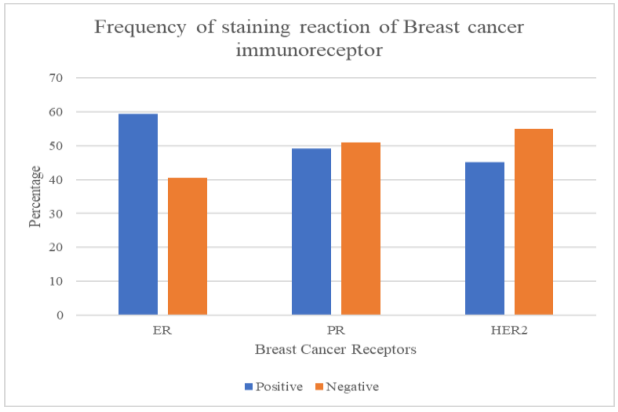

Figure 2. Frequency of staining reaction of Breast cancer immunoreceptor.

After investigation, the immunohistochemical pattern of breast cancer receptors and their molecular subtypes were evaluated respectively. Out of subjected 800 samples of breast cancer patients, 245 (30.6%) were suggestive of the Luminal A subtype, 234 (29.3%) were the Luminal B subtype. 136(17%) were HER 2 neu positive and 185 (23.1%) belonged to Triple - negative subtypes. The frequency of molecular subtypes of breast cancer is described (Figure 3).

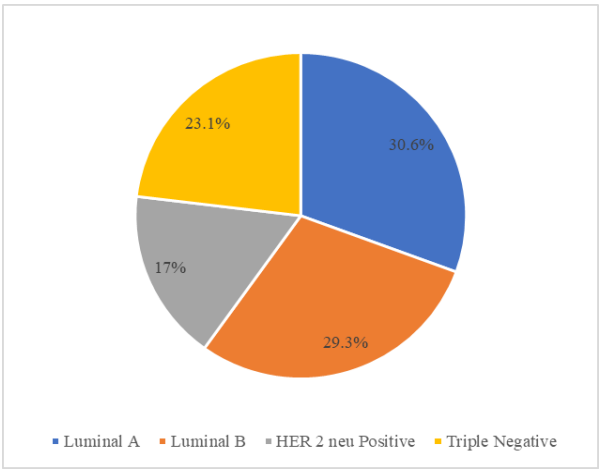

Figure 3. Frequency of Molecular Subtypes of Breast Cancer

Frequency of cancer differentiation of breast cancer is described (Figure 4). Out of 800 samples that were analyzed in this research 31 (3.9%) had good differentiation tumors,

437(54.6%) had moderately differentiated and 332 (44.5%) were lying in a poorly differentiated category.

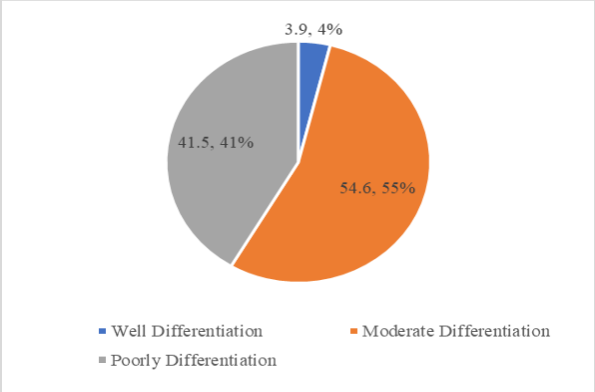

Figure 4. Tumor Differentiation of Breast Cancer.

A comprehensive clinicopathological correlation within molecular subtypes has laid out clinically useful patterns. Luminal A was followed by its 81% membership of premenopausal status, while Luminal B had 69%. The type that dominated postmenopausal was the Triple Negative subtype itself and upon confirmation overwhelmingly so with 92%. Family history of breast cancer was noted in 26% of Luminal A cases and 13% of Luminal B cases; it was absent in HER2-enriched cases and rare (3%) in Triple Negative patients. Tumor size varied among subtypes, with the largest average size being Luminal A (4.03 cm), followed by Luminal B (3.43 cm), Triple Negative (3.07 cm), and HER2-enriched (2.89 cm). In terms of lymph node involvement, HER2-enriched tumors most frequently had involvement (69%), followed by Triple Negative (63%), Luminal A (38%), and Luminal B (29%) (Table IV).

Table IV: Clinicopathological Correlation with Molecular Subtypes.

Feature	Luminal A	Luminal B	HER2-Enriched	Triple Negative
Premenopausal (%)	81	69	58	8
Postmenopausal (%)	19	31	42	92
Family History Positive (%)	26	13	0	3
Mean Tumor Size (cm)	4.03	3.43	2.89	3.07
Lymph Node Involvement (%)	38	29	69	63

Discussion

This study discovered a significant and noticeable increase in the disease's progression toward younger ages. The average age gap of the patients was 42.4 ± 12.6 years. This is a marked contradiction to the commonly cited incidence of 60 years old. According to the United State National Cancer Institute's surveillance epidemiology and Results (SEER) figures from 2003 to 2007, Just 1.9 percent of overall breast cancer patients was under 34 years, and 32 percent was between the age of 35 and 54 years. The average age was similarly 61. In the present study, disease onset at a younger age can be described by racial and social disparities. In comparison to my study, the United States has a longer mean expected life duration and more regularly utilized hormonal replacement treatment, leading to an increased average age for this illness. Women of the color of Asian descent get the disease at a younger age than white women. The increased prevalence of BRCA1 & BRCA2 alterations in the Pakistani community could have a role in the study's younger patients. Various Pakistani experts have also confirmed this phenomenon. Total of 800 patients were assessed in this study. The most common grade of breast cancer detected in our group was histological grade II, which accounted for 55.4percent of the total 800 samples. The molecular subtypes of breast cancer were examined after the analysis of the immunohistochemistry pattern of the receptors. There were 245 (30.6%) Luminal A subtype samples and 234 (29.3%) Luminal B subtype samples among the 800 breast cancer patient samples tested. 136 (17%) were Her 2 positive, and 185 (23.1%) were of the triple-negative subtype. The most prevalent tumor grade was moderately differentiated malignancy, which accounted for 437 of the 800 cases. Only 31 patients had well-differentiated tumors, while 332 had a disease that was poorly differentiated. The majority of the patients were in an advanced stage of disease until they were first assessed. This could be due to the study's increased number of patients with less differentiated tumors. This is an extremely awful condition that represents our society's illiteracy, destitution, and incompetence. Our conservative society's conventional “purdah” is also a major factor of consideration. The participation of homeopathic doctors, quacks, and Hakeem in immoral, nonscientific therapeutic practices cannot be overlooked. Numerous Pakistani researchers have indicated the delayed arrival of progressive disease in our nation. The immunohistochemical diagnosis was used to divide all breast cancer cases in four subgroups in this investigation, one of the most common molecular subtypes in the research was Luminal A, which accounted for 30.6 percent of all cases. Our findings support the findings of Yen Rahmawati, et al., who published molecular subtypes of Indonesian breast cancers and the lack of their association with patient age and tumor size, in which they revealed that luminal A was the most frequent molecular subtype of breast cancer in Indonesia.[11] In a study on molecular subtypes of breast cancer in Saudi Arabia Norah A. Alnegheimish et al. discovered that luminal A cancers were the most frequent form, followed by triple-negative cancers in their

community.[12] Mohammad Akbar et al. revealed that the most prevalent subtypes were triple-negative and HER2 neu-positive.[13] In contrast, Rubina Gulzar et al. did an immunohistochemistry assessment study on molecular subtypes of breast cancer and found that luminal B was the most frequently found molecular category in their community.[14] Most of these investigations revealed that the frequency of molecular breast cancer subtypes varies between population groups. This could explain why breast cancer patients in South Asian nations have a bad prognosis, as these cancers are more rapid and have high growth index. In a community cohort study titled as distinct Distribution and prognostic importance of molecular subtypes of breast cancer in Chinese Women, Yinghao Su et al. found that the triple-negative and HER 2overexpressed subsets were associated with impaired results in the luminal A group between females. The HER2-positive subgroup was more common in this Chinese community than in Western ones, indicating that systematic HER2 diagnosis and anti-HER? treatment could help a large number of Chinese women with breast cancer.[15] In 2009, Ching-Hung Lin et al. published molecular subtypes of breast cancer emerging in young women in Taiwan and proof that it is more than just Western influence as a justification for the illness in Asia, in which they stated that genetic Variants or relations of biological factors with other natural conditions may play a part to the oncogenesis of youthful women's breasts In Taiwan other Asia In breast tumors, they discovered a 63% incidence of luminal A subtype and an 8%Prevalence of basal-like subtype.[16]

The second most common subtype of research was Luminal B, which accounted for 29.3 percent of cases. Many international pieces of research, on the other hand, have found a substantially greater frequency of ER/PR-positive tumors. Madhuri kakarala and associates from the University of Michigan analyzed surveillance and epidemiology research data from 1988 to 2006 and found that the prevalence of estrogen receptor-negative breast tumor subgroup was substantially greater in Asian females than in European females.[17] An additional explanation for serious disease in our region is a greater incidence of estrogen receptor-negative tumors, which is another reason to push surveillance and awareness campaigns efforts to diagnose the disease at an initial phase. Breast cancer subsets based on estrogen receptor, progesterone receptor, and Her 2 neu expression and their correlation with clinicopathologic aspects and mortality by Adedayo A. Onitilo et al., found that the triple-negative type has the lowest average of disorder-free survival comparable to other subtypes.[18] To fine-tune the Immunohistochemical diagnosis, more research is required. Luminal B is linked to a younger patient's age, a greater grade, and a higher probability of lymph node metastases than Luminal A. Luminal A cancers only necessitate anti- endocrine medication, whereas luminal B cancers with a considerable relapse may improve from drug treatment. HER2 cancers react better to potential therapeutic anthracycline/taxane-based chemotherapy and Trastuzumab (Herceptin) than triple-negative breast cancers. TNBC is administered with drug treatment depending on anthracyclines and taxanes. Immunohistochemical stains should be widely utilized to identify luminal A and B tumors, according to the latest Saint Gallen consensus meeting, and multigene analysis such as Oncotype DX and Mamma Print may Provide valuable information.

Conclusions

This study assessed the frequency and clinicopathological correlations of molecular subtypes of breast cancer using immunohistochemistry in a cohort of 800 invasive ductal carcinoma cases. The findings lend strength to the assertion that breast cancer is a heterogeneous disease where distinct subtypes are defined by estrogen receptors, progesterone receptors, and HER2/neu expression. The most frequently occurring subtype was luminal A, followed by luminal B, HER2-enriched, and triple-negative subtypes. The largest proportion of cases fell within the age group 41-50 years with regards to sex, where middle-aged women mostly present the most common histological tumor grades as moderately differentiated grade histopathologically. However, more importantly, among younger patients, there was a significant proportion of high-grade tumors, which requires a higher level of awareness and involvement in early detection in this group. Therefore, this study confirms the utility of immunohistochemical subtyping in highly clinically relevant and cost-effective approaches to gene expression profiling, with more immediate implications for planning personalized treatment. Most importantly, the study offers valuable epidemiological data on breast cancer subtypes in the Pakistani population and emphasizes the relevance of molecular classification in informing targeted therapies and, therefore, improved patient outcomes.

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