



Immunohistochemical Marker Patterns in Female Breast Cancer

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Abstract

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BACKGROUND: Breast cancer (BC) represents the most common cancer in women worldwide and in Bulgaria. Its great medicosocial importance determines the intensive complex research devoted to BC prevention, early diagnosis, and management.

AIM: The objective of the present investigation is to reveal some essential peculiarities of four main immunohistochemical markers used in the diagnosis of molecular subtypes of female BC.

MATERIALS AND METHODS: During the period between December 1, 2017, and November 30, 2020, we examined a total of 128 randomly selected female BC patients operated on in Marko Markov Specialized Hospital for Active Treatment of Oncological Diseases of Varna, Bulgaria. We analyze BC molecular types and four immunohistochemical markers in BC patients. The expression of estrogen (ER) and progesterone (PR) receptors is assessed in mammary gland biopsies and surgical specimens using the indirect immunoperoxidase method with EnVision™ FLEX MiniKit, that of epidermal growth factor receptor 2 (HER2) with HercepTest™ and that of Ki-67 proliferation index with Leica Aperio Scan Scope AT2 device. The positivity and negativity of these receptors in single molecular subtypes are evaluated.

RESULTS: The luminal B HER2-positive and the luminal B HER2-negative subtypes are most common-in 36.72% and 35.16% of the cases, respectively. TNBC subtype is established in 11.72% the luminal A – in 8.59% and the non-luminal HER2-positive subtype – in 7.81% of the cases. The positive expression is statistically significantly more common in ER ($t = 8.972$; $p < 0.0001$) and PR ($t = 2.828$; $p < 0.01$), while the negative expression insignificantly prevails in HER2.

CONCLUSION: Our immunohistochemical results in female BC patients prove the role of single receptor expression for the proper and timely decision-making about the necessity and benefit of additional chemotherapy in selected surgically treated cases.

Introduction

Nowadays, breast cancer (BC) represents the most common cancer in women worldwide and in Bulgaria. The great medicosocial importance of this malignancy determines the intensity of complex research in different fields such as prevention, early diagnosis, and management.

BC molecular hallmarks include the immunohistochemical markers estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2), and proliferation marker protein Ki-67, as well as the genomic markers BRCA1, BRCA2, and PIK3CA [1].

During the past years, along with conventional BC immunohistochemistry, several modern methods are effectively used for the purposes of patients' proper diagnosis, post-operative therapy, and prognostication. Here belong the *in situ* hybridization, fluorescence, chromogenic, and dual *in situ* hybridizations as well as multiplex immunohistochemistry/immunofluorescence.

It is noteworthy that recently, a variety of modern imaging diagnostic methods are successfully applied in combination with immunohistochemistry in female BC patients.

The association between contrast-enhanced cone beam breast computed tomography features, immunohistochemical receptors, and molecular subtypes is retrospectively investigated in 240 invasive BCs of 211 women [2]. A multivariation logistic regression model reveals that BC size (odds ratio of 1.244), mass shape (odds ratio of 0.311), spiculation (odds ratio of 0.159), and internal enhancement pattern (odds ratio of 0.227) are related to differentiation between luminal and non-luminal subtypes (area under the curve of 0.809). Combined imaging features such as lesion type (odds ratio of 0.118), calcifications (odds ratio of 0.181), and degree of lesion enhancement (odds ratio of 0.962) are significant indicators of TNBC versus HER-2-enriched subtypes (area under the curve of 0.913).

A total of 151 female BC patients with 160 malignant lesions are examined within a retrospective double-center study between November 2017 and April 2020 using contrast-enhanced mammography and

immunohistochemistry of several molecular subtypes [3]. There is association between a higher standard deviation of lesion density and non-luminal ($p = 0.004$) and HER2-enriched BC ($p = 0.006$). The presence of calcification is associated with HER2-enriched BC ($p = 0.031$) and the presence of architectural distortion is related to luminal BC ($p = 0.010$) and non-TNBC ($p = 0.022$).

A developing data set and a test data set are generated from abnormal mammograms of BC patients from January 2006 through December 2016 and from January 2017 through December 2017, respectively, to train and validate the deep learning-based model for classifying the expression of positive and negative ER and PR as well as HER2-enriched and non-HER2-enriched [4]. Both sets include 1448 and 225 images, respectively. The analysis of the area under the curve for each receptor proves that this model effectively and noninvasively classifies the receptor expressions from the mammograms.

During a retrospective study of 289 breast tumors from 284 BC patients undergoing pre-operative ^{18}F -fluorodeoxyglucose positron emission tomography/computed tomography, 182, 24, 47, and 36 tumors are classified as hormonal, HER2, dual (with both hormonal and HER2 features), and TNBC subtypes, respectively [5]. The standardized uptake value significantly correlates with Ki-67 proliferation index expression level. However, it negatively correlates with ER ($r = -0.234$; $p < 0.001$) and PR expression ($r = -0.220$; $p < 0.001$).

Data from 248 consecutive invasive BC women at a mean age of 54.6 ± 12.2 years undergoing dynamic contrast-enhanced magnetic resonance imaging and diffusion-weighted imaging between 2019 and 2020 are retrospectively evaluated [6]. Sixty-one (24.60%) patients are classified as luminal A subtype, 130 (52.42%) as luminal B subtype, 25 (10.08%) as HER2-enriched, and 32 (12.90% of the cases) as TNBC. There are statistically significant differences in the kinetic and apparent diffusion coefficient heterogeneity values among molecular BC subtypes ($p < 0.001$ and $p = 0.023$, respectively). The multivariation linear analysis indicates that the HER2-enriched ($p < 0.001$) and TNBC subtypes ($p < 0.001$) are statistically significantly associated with higher kinetic heterogeneity values as the TNBC subtype ($p = 0.042$) is significantly related to the higher apparent diffusion coefficient heterogeneity values, too [6].

The results from a retrospective study of 115 patients with suspicious breast lesions undergoing breast magnetic resonance examinations (including synthetic magnetic resonance imaging mappings) carried out between May 2019 and October 2020 demonstrate that T2 and T2-Gd can differentiate luminal A/B subtypes of BC from non-luminal ones ($p = 0.005$ and $p = 0.015$, respectively) [7]. T1 and T2 values are higher for TNBC and lower for non-TNBC. T2 and T2-Gd values are lower for luminal A/B BCs and higher for non-luminal ones.

The objective of the present investigation is to reveal some essential peculiarities of four main immunohistochemical markers used in the diagnosis of molecular subtypes of female BC.

Materials and Methods

During the period between December 1, 2017, and November 30, 2020, we examine a total of 128 randomly selected female BC patients at a mean age of 59.48 ± 11.99 years (range, 30–84 years) operated on in Marko Markov Specialized Hospital for Active Treatment of Oncological Diseases of Varna, Bulgaria.

We analyze BC molecular types and these four immunohistochemical markers in BC patients. The expression of ER and PR is assessed in mammary gland biopsies and surgical specimens using the indirect immunoperoxidase method with EnVision™ FLEX MiniKit (HighpH, DAKO Denmark A/S), that of HER2 with HercepTest™ (DAKO Denmark A/S) and that of Ki-67 proliferation index with Leica Aperio Scan Scope AT2 device (AperioTechnologies, Vista, CA, USA) [8]. A special attention is paid to the positivity and negativity of these receptors in single molecular subtypes.

Results

The immunostaining histochemical findings of the positive expression of the ER, PR, and HER2 and of the Ki-67 proliferation index are demonstrated in Figures 1-4.

Among our patients, both luminal B HER2 subtypes, the positive and negative one, are most common – in 36.72% and 35.16% of the cases, respectively. TNBC subtype is less frequently

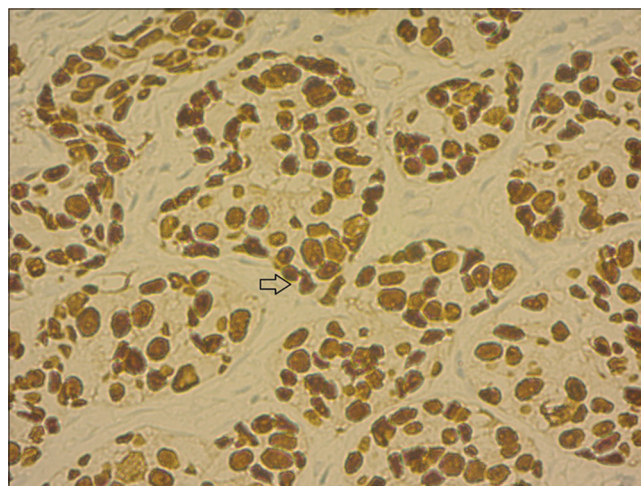


Figure 1: Positive ER expression (2+). Staining with DAKO. Magnification $\times 400$. Arrow indicates stained malignant cells

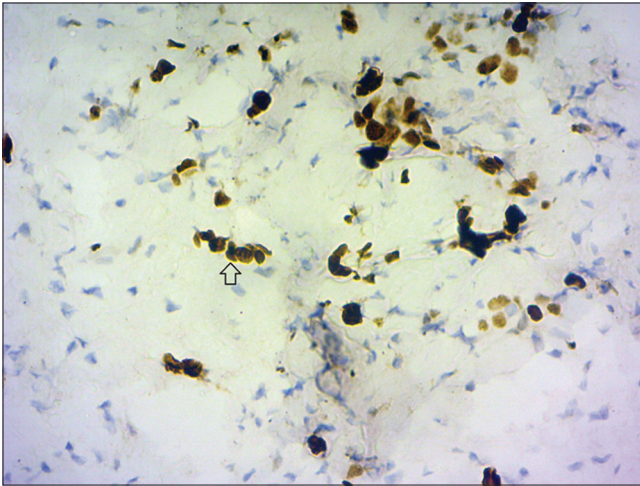


Figure 2: Positive PR expression of 80% (2+). Staining with DAKO. Magn. $\times 400$. Arrow shows stained malignant cells

established (in 11.72%) followed by the luminal A (in 8.59%) and the non-luminal HER2-positive subtype (in 7.81% of the cases).

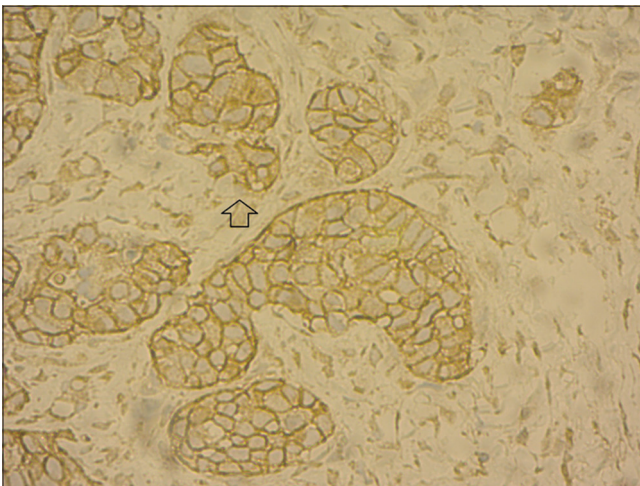


Figure 3: Positive HER2 expression. Staining with DAKO. Magn. $\times 400$. Arrow demonstrates stained malignant cells

Patients' distribution according to five BC molecular subtypes is presented in Figure 5.

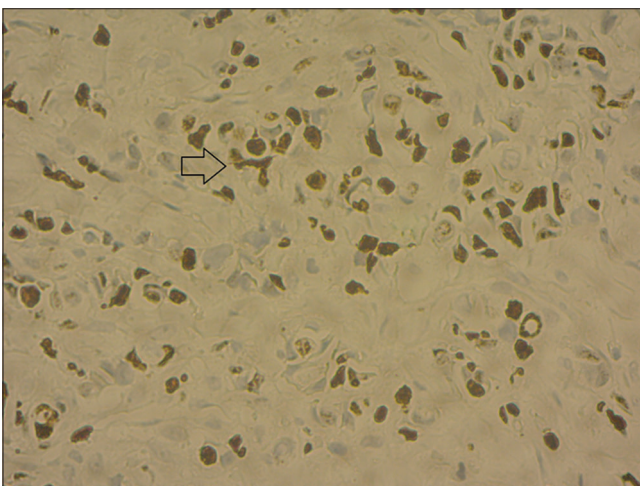


Figure 4: Strongly positive Ki-67 expression of 50%. Staining with DAKO. Magn. $\times 400$. Arrow displays a group of stained malignant cells

In Table 1, patients' distribution according to age groups and BC molecular subtype is shown.

The number of the patients with minimal, maximal, and mean age values according to BC molecular subtype is displayed in Table 2.

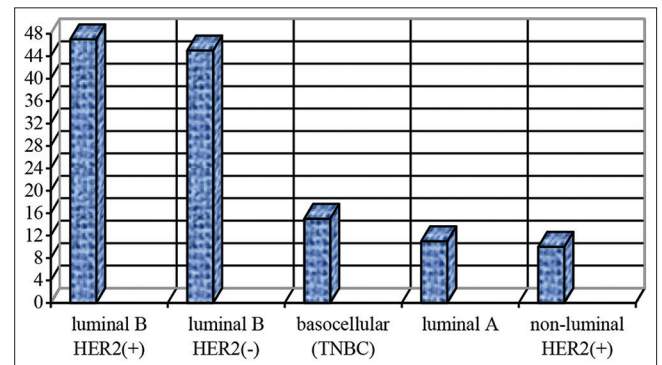


Figure 5: Patient's distribution according to BC molecular subtype

All patients' distribution with positive and negative receptor expression is demonstrated in Table 3.

The patients with positive ER and PR strongly prevail when compared to those with negative ones. These differences are statistically significant in terms of ER ($t = 8.972$; $p < 0.0001$) and PR ($t = 2.828$; $p < 0.01$). HER2 negativity insignificantly prevails over HER2 positivity.

The results from the analysis of the distribution of the patients presenting with negative ER, PR, and HER 2 according to BC molecular subtypes are illustrated in Figures 6-8. There are 12 patients with negative ER, 33 patients with negative PR, and 57 patients with negative HER2.

On the other hand, 31 patients (24.22% of all the cases) present with positive receptors only (Figure 9). There is a very strong domination of the luminal B HER2-positive molecular subtype (in 93.55% of these cases).

Discussion

The review of the most recent publication on this hot topic reveals certain interesting peculiarities concerning the distributions of the various molecular subtypes among BC females worldwide.

The most common molecular subtypes among 222 BC patients examined by immunohistochemistry of core needle biopsies and/or surgical specimens are luminal A (in 43.2%) and luminal B HER2-negative (in 29.7% of the cases) [9].

Table 1: Patients' distribution according to age groups and BC molecular subtype is shown

Molecular subtype	Age groups					Total
	31-40 year	41-50 year	51-60 year	61-70 year	71-80 year	
luminal B HER2 (+)	5	8	9	14	9	47
luminal B HER2 (-)	2	10	13	10	6	45
basocellular (TNBC)	1	4	4	3	3	15
luminal A	0	1	3	4	3	11
non-luminal HER2 (+)	0	4	2	2	2	10
Total	8	27	31	23	23	128

Table 2: Patient's minimal, maximal, and mean age values according to BC molecular subtype

Molecular subtype	Minimal age	Maximal age	Mean ± SD
luminal B HER2 (+)	30	84	59.68 ± 13.04
luminal B HER2 (-)	34	84	59.78 ± 12.96
basocellular (TNBC)	32	78	56.27 ± 12.83
luminal A	41	78	63.45 ± 11.62
non-luminal HER2 (+)	43	79	57.60 ± 12.60
Total	30	84	59.48 ± 11.99

The analysis of female BC patients' distribution according to favorable (≥ 5) and unfavorable (< 5) hormone receptor expression values among 113 BC patients demonstrates that the number of ER with values ≥ 5 is by 5.65 times greater than that of ER with values < 5 , the number of PR with values ≥ 5 is by 1.63 times only greater than that of PR with values < 5 , while the number of the HER2 with favorable values of 2 and 3 is almost equal to that of the HER2 with unfavorable values of 0 and 1 [8].

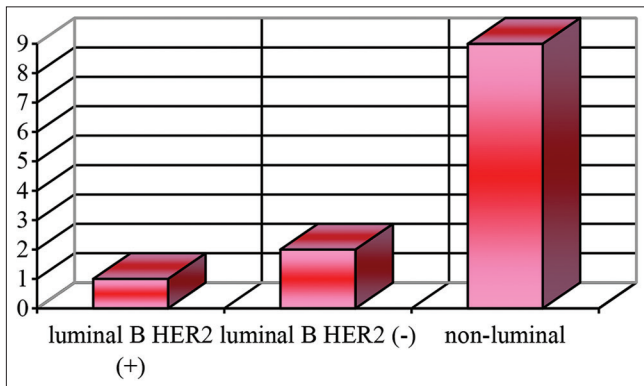


Figure 6: Patient's distribution with negative ER according to three BC molecular subtypes

The results from a systematic review and meta-analysis of 16 case-control and four cohort studies retrieved from eight mainstream databases up to January 12, 2021, show that among 1135131 participants, there are 7795 luminal A, 3576 luminal B, 1794 overexpressing HER2-negative, and 5192 TNBC cases [10]. The combined odds ratios for ever parity versus nulliparity indicate a reduction by 34% of the luminal A subtype risk and a by 29% of the luminal B subtype risk. In the dose-response analysis, there is a potentially non-linear and gradually increasing protective relationship between the number of parity and luminal BC subtype risk.

Table 3: Patients' distribution according to the number and relative share of positive and negative receptor expression

Expression	Receptors					
	ER		PR		HER2	
	N	%	n	%	n	%
Positive	101	78.02	80	62.50	56	43.15
Negative	27	21.98	48	37.50	72	56.85
Total	128	100.00	128	100.00	128	100.00

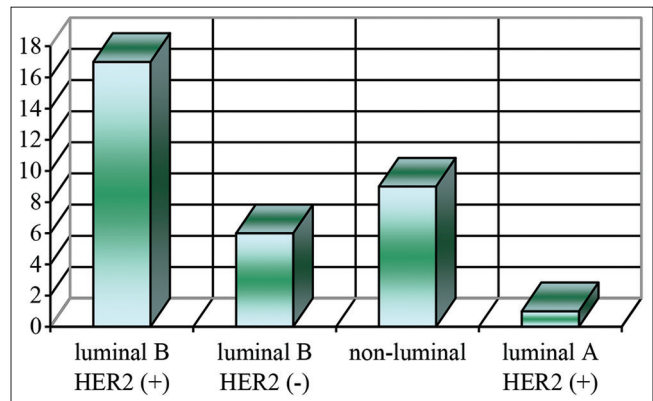


Figure 7: Patient's distribution with negative PR according to four BC molecular subtypes

By means of immunohistochemistry on tissue microarrays for cytokeratin 5/6 and 4/14, epidermal growth factor receptor, vimentin, E-cadherin, claudin 3 and 7, androgen receptor, and aldehyde dehydrogenase 1A, 245 TNBCs are classified into six major subtypes [11]. They are the following: Basal-like one subtype (in 32 patients or in 13.1%), basal-like two subtype (in four patients or in 1.6%), mesenchymal subtype (in 70 patients or in 28.6%), luminal androgen type (in 41 patients or in 16.7%), mixed subtype (in 37 patients or in 15.1%), and unclassified sub type (in 61 patients or in 24.9% of the cases).

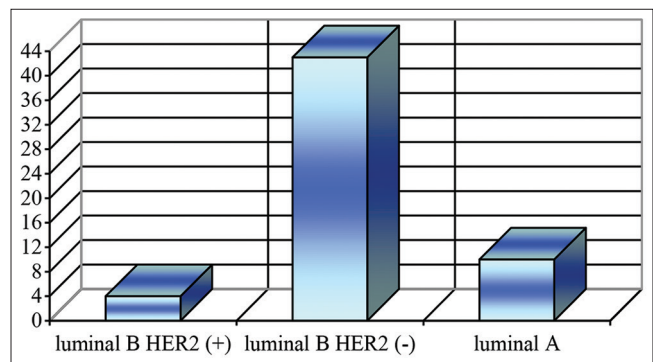


Figure 8: Patient's distribution with negative HER2 according to three BC molecular subtypes

Among 195 HER2 fluorescence *in situ* hybridization-equivocal BC samples collected between 2014 and 2018, 183 (93.85% of the cases) are classified as luminal-like subtype [12]. According to 2018 ASCO/CAP guideline, all these cases are recategorized as HER2 negative. Some 152 BC cases (77.95%) are luminal B-like (HER2 negative), 31 cases (15.90%) are luminal A-like, and 12 (6.15%) are triple negative.

Within a retrospective study of 432 female BC patients from a tertiary care center of Northeast India,

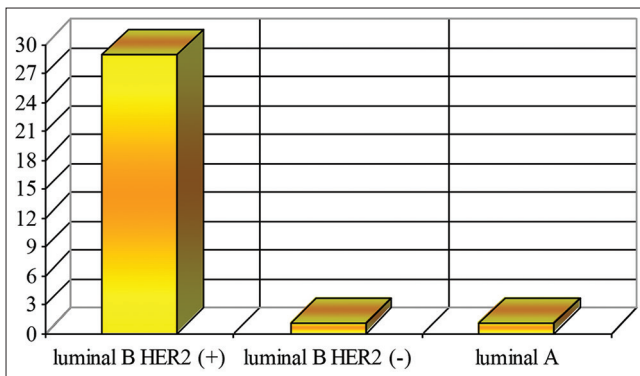


Figure 9: Patient's distribution with positive receptors only according to BC molecular subtypes

immunohistochemistry indicates that luminal B is the most common molecular subtype (in 31%) in younger women aged ≤ 40 years followed by TNBC (in 20%), luminal A (in 14%), and HER2 (in 5.3% of the cases) [13]. In older women aged > 40 years, the luminal B subtype prevails, too (in 27.8%) followed by TNBC (in 14%), HER2 (in 12.2%), and luminal A subtype (in 12% of the cases).

The results from a retrospective, cross-sectional, and descriptive study of 222 BC women at a median age of 54.8 years (range, 25–91 years) performed in Mankweng Hospital Breast Oncology Clinic in Limpopo Province, South Africa, demonstrate that luminal B is the most predominant molecular subtype (in 107 patients or in 48.19%) followed by luminal A (in 51 or in 22.97%), TNBC (in 38 or 17.12%), and overexpressed HER2 (in 11 or in 26.75% of the cases) [14]. There are 90 HER2-positive (40.54%) and 132 HER2-negative patients (59.46% of the cases).

The results from a retrospective and cross-sectional study of 379 BC patients at a mean age of 54.63 years (range, 23–89 years) in Mexico indicate luminal B subtype in 143 (in 37.73%), luminal A subtype in 139 (in 36.67%), TNBC in 65 (in 17.15%), and HER2 (+) in 32 patients (in 8.44% of the cases) [15].

The analysis of the national health registries of immigrants in Norway between 2005 and 2015 establishes a lower incidence rate of the luminal A-like subtype among invasive BC female immigrants from Sub-Saharan Africa, South East Asia, South Asia, and Eastern present with higher incidence rate ratio rates of HER2-positive BCs [16]. Women from Eastern Europe, Sub-Saharan Africa, and Asia have different subtype-specific incidence rates when compared to women from high-income countries (including non-immigrants).

Molecular subtyping is performed in 124 of 152 TNBC tumors from a prospective, multicenter cohort of histopathologically confirmed invasive and non-metastatic BCs [17]. There are 23 TNBCs identified as luminal androgen receptor subtype. After standard adjuvant or neoadjuvant chemotherapy, these patients show the most events for 5-year recurrence-free interval survival and the poorest probability of 5-year

overall survival when compared to those with non-luminal androgen receptor disease.

The flowcytometric analysis of the expression distribution of cancer stem cell phenotype indicates that there is a highest population of these cells in the luminal B subtype (in 3.4%) [18]. Next come TNBC (in 1.7%), HER2 (1.6%), and luminal A subtype (in 1.3% of the cases).

Immunohistochemistry on tissue microarrays containing a cohort of 361 luminal subtype BC reveals low expression levels of RET, BCAR1, and BCAR3 genes [19]. BCAR3 expression correlates with response to hormonal therapy ($p = 0.021$) and with poor prognosis ($p = 0.042$). These three genes are potential candidate markers for endocrine therapy resistance in luminal BC patients.

Among 300 invasive BC patients at an average age at time of diagnosis of 44 years, TNBC is the most common molecular subtype (in 34.3%) followed by luminal B (in 33.4%), luminal A (in 17%), and HER2 positive subtype (in 15.3% of the cases) [20].

The role of Ki-67 proliferation index with cutoff value of 14% in molecular subtypes and its association with patient's prognosis is evaluated by immunohistochemical staining in 278 histopathologically confirmed BC sections in Pakistan [21]. High Ki-67 proliferation index expression in 88% of the cases is significantly associated with immunoeexpression of ER, PR, and HER2. The luminal B subtype is identified in 51%, TNBC – in 20%, HER2 enriched – in 18%, and luminal A – in 10% of the cases. The Ki-67 proliferation index is significantly high in 98% of HER2 enriched and 95% of TNBC patients.

The investigation of ribonucleoside-diphosphate reductase small subunit protein expression using immunohistochemistry and tissue microarrays in Egyptian women with BC identifies a positive expression in about 77% of the cases [22]. Most non-luminal cases express this protein. There is high Ki67 proliferation index among the cases with high score of this protein. In ER positive patients, the expression of the ribonucleoside-diphosphate reductase small subunit is associated with shorter disease-free survival with borderline significance.

During the investigation of the effect of long-lasting neoadjuvant endocrine therapy with aromatase inhibitors in postmenopausal primary ER-positive BC patients, tumor transcriptional profiles undergo considerable changes in terms of intrinsic molecular subtypes [23]. Most luminal B subtype cases and one-half of HER2 enriched cases at baseline are reclassified as luminal A or normal-like subtype after this therapy.

Between 2013 and 2018, a total of 130 BC patients in Taipei Veterans General Hospital receive molecular subtyping testing [24]. This testing restratifies 44 tumors as subtype shifting includes 20 ones from luminal A to luminal B and 24 ones from luminal B to

luminal A intrinsic subtypes. These results dominate decision-making of adjuvant therapy.

Survival analysis in 387 luminal B (HER2-negative) and 82 luminal B (HER2-positive) BC subtypes demonstrates that positive PR expression is statistically significantly more frequent concerning distant recurrence rate than mortality rate (86.3% versus 61.0%, respectively; $p < 0.001$) [25]. In univariate analysis only, PR expression is a significant favorable prognostic factor for distant disease-free survival and overall survival in both subtypes.

Conclusion

Our results from the immunohistochemical study of female BC patients prove the role of single receptor expression for the proper and timely decision-making about the necessity and benefit of additional chemotherapy in selected surgically treated cases. There is a need for the wide application of additional immunohistochemical biomarkers already available such as cisplatin and of several modern immunohistochemistry methods such as fluorescence, chromogenic, and dual *in situ* hybridizations to make the most optimal individualized therapeutic decision and warrant the best possible prognosis.

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