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Molecular Subtypes by Immunohistochemical for Iraqi Women with Breast Cancer

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Abstract: Breast cancer is a complex disease encompassing multiple tumor entities, each characterized by distinct morphology, behavior and clinical implications. Hormone receptor status and HER2 status are of critical interest in determining the prognosis of breast cancer patients. Their status is routinely assessed by immunohistochemistry (IHC). The aim of this study is to clarify the differences in the expression of the established prognostic and predictive markers ER, PR and HER2 with breast cancer by IHC. This study was conducted at AL-SADER Hospital in AL-Najaf and Medical city in Baghdad, Iraq, from July 2018 to April 2019. It included fresh tissue of 48 patient women with breast cancer. Immunohistochemical staining with antibodies for Estrogen Receptor (ER), Progesterone Receptor (PR) and Human Epidermal Growth factor 2 (Her 2) was performed and then breast cancers were classified into four molecular subtypes: Luminal A (ER/PR +, HER2-), Luminal B (ER/PR +, HER2+), Triple Negative Breast cancer (Basal like) (ER/PR -, HER2-) and HER 2 (ER/PR -, HER2+). Clinical parameters were compared using chi-square test. In the current study ER receptors was 72.91% (35/48), PR positive receptors 56.25 % (27/48) and that 11 out of 48 malignant cases were represent as 22.91 % were positive for her2/neu expression. Corresponding for the hormones statues, the molecular subtype was: Luminal A group 56.25% (27/48), Luminal B subtype which was 16.66% (8/48), then a group with triple negative (ER-, PR- and HER2-) was 18.75% (9/48) cases that represent Basal like group, while the other groups appeared least frequency as a following: HER +, ER- and PR- were (4) cases only which were referred to HER2 subtype group. There is certain variation among the molecular subtype of breast cancer with prevalence of certain types among different regional population.

Keywords: ER, PR, HER2, breast cancer, IHC.

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Introduction

Molecular subtypes, originally identified by gene expression profiling (1,2,3,4) were then confirmed by IHC analysis(5,6). Numerous studies have shown that Luminal A subtype has better differentiated tumors, is often seen in older patients and has the best prognosis compared to other subtypes. Luminal B subtype has higher expression of proliferation associated genes and a worse prognosis than Luminal A. HER2 subtype is often associated with nodal metastasis and Basal subtype often occurs in younger patients, is more frequently associated with visceral organ metastasis and has a poor prognosis(7,8). Since molecular classification has been shown important clinical implications for breast cancer patients. It is important to understand that in spite of IHC-based molecular classification has been used in

numerous studies. There is still lack of uniform definition for each subtype and the definitive role of molecular classification in guiding clinical decision making remains to be confirmed (9,10). Estrogen receptor (ER) and progesterone receptor (PR) are hormone receptors found on breast cells that pick up hormone signals resulting in cell growth. Similarly, positive human epidermal growth factor receptor-2 (HER2/neu) status of the breast carcinoma means that HER2/neu gene is making too many HER2/neu proteins, which acts as receptors on the cell surface and helps the cells to grow and divide (11). Hormone receptor studies such as ER, PR, and HER2/neu are routinely done in breast carcinoma. It not only helps in the prognosis of the tumor but also helps in deciding its treatment. The goal of doing this receptor status is to provide right treatment to the right patient. This hormone receptor status is graded using Allred scoring and grading system. Depending on the hormone status, breast carcinomas can be divided into a number of different categories ranging from triple positive through triple negative. Another marker of proliferation (Ki-67) is also being used which is a proliferation index marker. This scoring system has its own limitation and shortcomings, which depends on a lot of pre- and post-analytical factors (8). Certain new techniques such as genomic assays, PAM50, and HALO screening test are being used nowadays for breast cancer detection. Biomarkers can be prognostic, predictive, or both. Prognostic biomarkers measure prognosis independently of other factors. The presence or absence of these biomarkers is directly associated with disease recurrence or mortality. Predictive biomarkers, on the other hand, predict whether or not a patient will respond to a given therapy. The presence of the hormone receptors ER, PR in a patient's breast cancer is an example of a weak prognostic but strong predictive biomarker. If a patient's tumor expresses ER and/or PR, we can predict that this patient will positively benefit from endocrine therapy such as tamoxifen. The overexpression of the oncogene HER2/neu in a patient's breast cancer is an example of both a prognostic and predictive biomarker. HER2/neu expression is associated with poor prognosis (high risk of recurrence [ROR]); however, it also predicts that a patient will more likely benefit from anthracycline and taxane-based chemotherapies and therapies that target HER2/neu (trastuzumab), but not to endocrine-based therapies (12,13).

Material and Methods

Forty Eight fresh malignant breast tissues with surrounding normal area samples were also collected. Each fresh tissue samples was divided to three parts, malignant tissue part, normal tissue part as control and malignant tissue part for immunohistochemistry assay. Parts were washed with sterilized normal saline and stored with 5 volumes of RNA *later*® Solution Tissue Collection: RNA Stabilization Solution (Ambion, Thermo Fisher, USA) until used in DNA, RNA extraction and immunohistochemistry assay. Fresh tissues samples were collected from some private hospitals in AL-Najaf AL-ASHRAF and Medical City of Baghdad between September 2018 - March 2019. Patient’s age ranging from 27-91 years old.

Four µm sections of multi-block were taken by ELICA Microtome fixed in slides. The immunosatining method used in the current study was Labeled Strept-Avidin Biotin (LSAB+) technique which was applied for HER2, ER and PR staining according to manufacture protocol of DAKO company. This system considers both the proportion and intensity of stained cells. The intensity score (IS) ranges from 0 to 3, with 0 being no staining, 1 weak staining,2 intermediate staining, and 3 intense staining. The proportion score (PS) estimates the proportion of positive tumor cells and ranges from 0 to 5, with 0 being non-reacting, 1 for 1% reacting tumor cells, 2 for 10% reacting tumor cells, 3 for one-third reacting tumor cells, 4 for two-thirds reacting tumor cells, and 5 if 100% of tumor cells show reactivity. The PS and IS are added to obtain a total score (TS) that ranges from 0 to 8.Tumor cells with a total score of 3 to 8 were considered positive, whereas those with a TS less than 3 were considered negative cases.Her-2/neu was scored on a 0 to 3 scale according to the criteria set by Dako. The staining was scored as: negative (0) when no membrane staining was observed, or when membranous staining was observed in less than 10% of the tumor cells; weak positive (1+) if weak focal membrane staining was seen in more than 10% of the tumor cells; intermediate (2+) if weak to moderate, complete membrane staining was seen in more than 10% of the tumor cells; and strongly positive (3+) if intense membrane staining with weak to moderate cytoplasmic reactivity was seen in more than 10% of the tumor cells. Figure 1 illustrates scores 1+, 2+, and 3+ as uses in this study. In the final analysis, however, scores 0 and 1 were considered negative; score 2 was considered weakly positive; and score 3 was considered strongly positive. Only score 3 cases were considered as Her-2 over expressing cases. The Statistical Analysis System- SAS (2012)(14) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability in this study.

Results and Discussion

The study revealed that more than 24% of the patients have family history either it is the first or second degree. The histopathology diagnosis as shown in table (1), showed a low percentage in Iraqi cases of the invasive lobular carcinoma represented (6.25%) while a high percentage with infiltrated ductal carcinoma represented (93.75%) with P-value 0.0001 (high significant) which agreed approximately well with those obtained by Groheux *et al*., (2013)(15). In current study 72.9 % of patients were in grade II and 27.1 % were in grade III (P-value 0.0001 high significant). The education levels of the patients showed that non-educated was 26/48 (54.17%) with P-value 0.0038 that appear significant value, but no significant between primary and secondary education 10/48 (20.83%), 12/48 (25.00%) respectively. No significant results appeared in the location of the tumor if it found in the right or left breast (P-value 0.0981). The results also revealed that breast cancer distributed in married patients (45-93.75%) more than non-married (3-6.25%) with P-value 0.0001. The median patient age was 50 (range 27 to 91 years) and the peak age frequency in the total group studied was (40-49 yr) 31.25% accounting of 48 patients as shown in table (2) followed with (50-59) 29.16 with highly

significant by Chi-Square ($\chi 2$) 9.026 P≤0.01) while the second groups were less frequency were: (30-39) 22.91% and 60-69) 18.75% , then the groups: (70-79), (20-29), 80≥ and (10-20) were 6.25%, 2.20%, 2.20% and 0% respectively. The present results revealed that a high age frequency of cancer occurred between (40-59)(P≤0.01). These results are corresponding with other demographic Iraqi studies revealed that the age range (40-59 years) accounting for (67/216) (31%) breast cancer patients was the most frequent in Iraq.

Table (1): Main tumor Clinic pathological data in the patients with breast cancer

Clinic pathological Features	No (%)	P-value
Age (years)		
Mean	50	---
Minimum	27	
Maximum	91	
Marital Status		
Married	45 (93.75%)	0.0001 **
Non- Married	3 (6.25%)	
Level of education		
Non-Educated	26 (54.17%) a	0.0038 **
Primary-Educated	10 (20.83%) b	
Secondary-Educated	12 (25.00%) b	
Breast Type		
Ductal	45 (93.75%)	0.0001 **
Lobular	3 (6.25%)	
Grade		
II	35 (72.92%)	0.0001 **
III	13 (27.08%)	
Location		
Lateral, right	23 (47.92%)	0.0981 NS
Lateral, left	25 (52.09%)	
**** (P≤0.01), NS: Non-Significant.**		

Table (2): Distribution of sample study according to age groups

Age group	No. of patient	Percentage
10-20	0	0.00 c
20-29	1	2.20 c
30-39	11	22.91 b
40-49	15	31.25 a
50-59	14	29.16 a
60-69	9	18.75 b
70-79	3	6.25 c
80≥	1	2.20 c
Chi-Square (χ^2)	----	9.026 **
**** (P≤0.01).**		

In another study by Majid *et al.*, (16), there was a significantly increased breast cancer rate between 2006 and 2012 among women ≥ 60 years old (P <0.001). The current results also agreed with Iraqi study by done by AL-Nuaimy *et al.*, (2015)(17) which used two groups (≤50 and 50 ≥ yrs) mentioned that the age group (40-49yrs) and (50-59yrs) were (47/70) 36.7%, (37/58) 29% respectively. A recent Iraqi study about breast cancer by done by AL-

Alwan *et al.,* (2019)(18) agreed with current study. The study revealed that the high frequent age groups were (35-49), (50-64) 42.4%, 42.2% respectively. Moreover, a group of researchers in both USA and Australia have found that the breast cancer incidence increase tremendously after the age of 40 (19,20). While some studies showed that the breast cancer incidence increased between 51-60yrs (38.89%) (21). The results of our study enforce the fact that the change in the hormonal factors in female play a key role in the behavior of the tumor corresponding to this age. Tumor showing positive receptors has better prognosis and better response to hormonal therapy than those with no receptors (21). ER, PR and HER2 were biogenic factors which were important in the early stage of breast cancer (22). This study demonstrated 48 malignancy breast carcinoma samples were included fresh tissues then confer to wax blocks embedded tissue to use in immunohistochemistry study.

Immunohistochemical profiles of these 48 carcinoma (Table 3) (Figure 1) showed that ER receptors were in 72.91% (35/48) of the cases and PR positive receptors in 56.25 % (27/48) of the cases so we concluded that there was hormone receptor expression in the majority of breast cancer in Iraqi patients under study and breast cancer was considered hormone receptor positive also they were likely respond to hormonal therapies. The study was focused on the relationship between the hormonal receptors and Her-2/neu status as Molecular subtype classification and subsequently correlated the results with the studied of clinical and morphoclinical parameters. In the current study determined that all of the cases with positive Her-2/neu of score 3+ were characterized by the absence of nuclear stain for both estrogen and progesterone receptors. Analysis of the relationship between the response to hormonal receptors and Her-2/neu status allowed the distribution of the breast cancer cases into molecular classification, using "surrogate Immunohistochemical criteria". 6 cases (12%) with equivocal Immunohistochemical stain (Her-2/neu score 2+) were excluded. In regard to her2/neu, the present study demonstrated that 11 out of 48 malignant cases were represent as 22.91% were positive for her2/neu expression, while 37 cases out of 48 were with score 0 and score1 and 6 cases with score 2 considered (equivocal) as her2 negative result (table -4). This results appear to be compatible well with that reported rates of 20 % to 30 % by (23-25) but more higher than that completely reported by other workers (26,27). Clinical utility of some parameters such as tumor size, histologic type and grade in addition to the hormone receptor and HER2 status of a primary breast carcinoma are important in determining patient treatment options and overall prognosis (28-32). This is similar to Mouttet, *et al.,* (2016)(33) study that showed the ER Immunohistochemistry analysis was 86% of the tumors were classified as ER-positive (140/163). 142 out of 161 tumors were classified as genomic ER-positive (88%), 6% of the tumors were HER2-positive (10/163)and 11 out of 161 tumors were classified as genomic HER2-positive (7%). Dai, *et al.,* (2016)(34) study explain that when the parameters taken together, in assessment of breast cancer variants are better than using either one alone.

Table (3): Molecular subtypes with their main tumor Clinicopathological for breast cancer patient women

Age Groups	No. of patients	Breast Type		Location of tumor		Molecular Subtype			
		Ductal	Lobular	Right	Left	Luminal A	Luminal B	Basal like	Her2
⩽30	1	1	-	-	1	-	-	-	1
30-39	11	11	-	6	5	7	1	4	-
40-49	15	13	2	6	7	7	2	2	1
50-59	14	14	-	7	6	9	1	1	1
60 ⩾	13	12	1	4	6	5	3	2	1

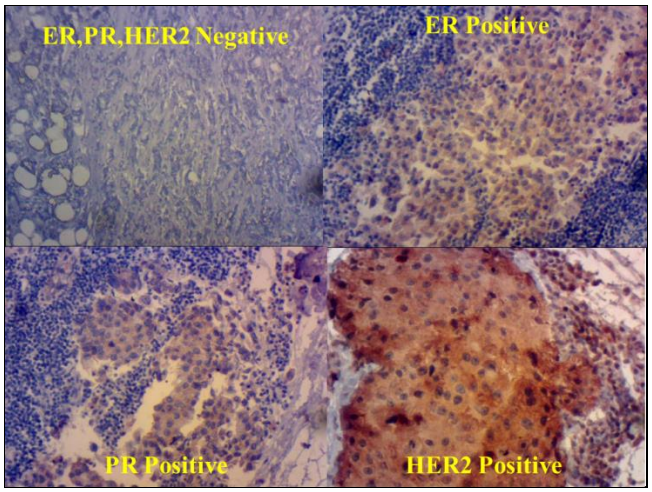

Figure (1): Representative staining results from H&E and IHC for Estrogen, Progesterone and HER2 receptor in malignant breast cell.

Table (4): Distribution of sample study according to results of Hormones

Hormone	No. of Positive	%	No. of Negative	%	P-value
ER	35	72.91	13	27.08	0.0001 **
PR	27	56.25	21	43.75	0.0274 *
Her2	11	22.91	37	77.08	0.0001 **
*** (P≤0.05), ** (P≤0.01).**					

According to wide American study during a median follow-up of 8.3 years, 18 586 women developed invasive breast cancer, they had recorded 14 969 ER-positive (80.5%) and 3617 ER-negative (19.5%) (35).

The results were compatible with the Iraqi cancer therapy registry (36) findings. They found that ER positive tumors were mentioned as a 65% of the breast cancer cases with PR positive tumors in 45% of the cases. In a study on hormone receptor contents of breast carcinoma specimens belong to Iraqi patients reported higher frequencies for ER and PR equivalent to 61%, 52% and 67.8%, 65.3% respectively (18,37). Other study detect elevated levels of estrogen in 19 cases (63%) (36) and in 26 (50%) cases (38). Our results also close to the rates that mentioned by Al-Nuaimy *et al.*, (2015)(17) who improved that each of hormonal receptors (ER and PR) were expressed in 63.3% of the cases, while HER-2/neu over expression was found in 18% of the cases. In AL-Bahrain, Almarzooq, *et al.*, (2018)(39) who got this result:

(65.7%) were ER-positive tumors, (57.8%) PR receptors were positive and (31.1%) patients had HER2 amplification. One the other hand Jordanian study(40) found that 50% and 57% of breast cancer samples were positive for ER and PR respectively. According to the classification of molecular subtype of breast cancer , the results showed that patients group - Luminal A -with ER+, PR+ and HER2- represent as 56.25 % (27/48) cases which highly significant comparing to other groups (Chi-Square (χ^2) 10.735). While the lowest group represented HER2 subtype (ER or PR is negative and HER2+) is 8.33% (4/48) (table-5) and (figure-2).

Table (5): Classification of Hormones according to Molecular Subtype

Subtype	ER and/or PR	HER2 overexpression	No. of Cases	%
Luminal A	+	-	27	56.25 a
Luminal B	+	+	8	16.666 b
HER2 subtype	-	+	4	8.333 b
Basal like	-	-	9	18.75 b
Total			48	100
Chi-Square (χ^2)				10.735 **
**** (P≤0.01).**				

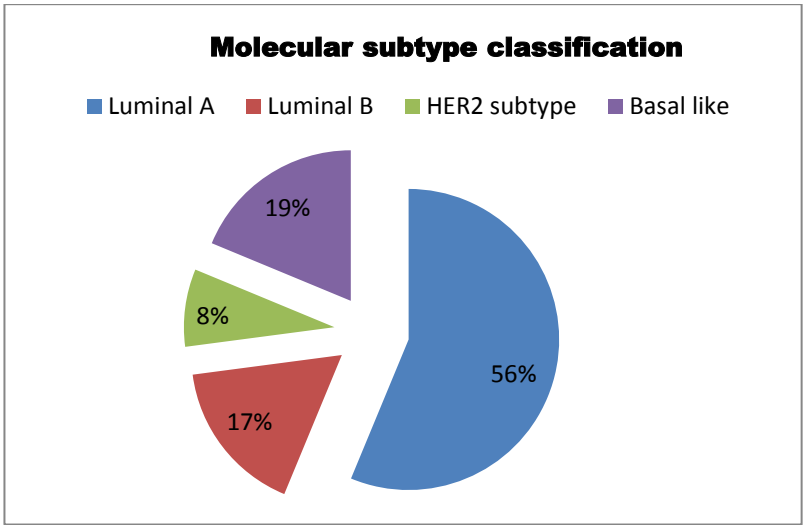

Figure (2): Molecular subtype classification.

These results were similar to Iraqi study by Al-Nuaimy *et al.*, (2015)(17) which obtained 72/128 (56.25%) of luminal A subtype, 9/128 (7.03%) of luminal B subtype, 14/128 (10.93%) of HER2 subtype and 33/128 (25.78%) of Basal like subtype. With a little differences with (17,18,41) mentioned that Immuno-histochemical evaluation revealed ER+PR+Her2+ (Triple Positive/Luminal B), ER-PR-Her2- (Triple Negative), ER+PR+Her2- (Luminal A) and ER-PR-Her2+ (Her2) subtypes were 13.4%, 11.8%, 48.2% and 9.8% of the examined breast samples respectively. Other studies showed that the differences in the subtypes between breast cancer women could be due to ethnic group where subtypes in Arabic women with luminal A and luminal B were 51.7% , 46.8 % respectively while in Kurdish women were 49.5% luminal A and 47.7% luminal B (16). While in

Pakistani study were very different result when Gulzar *et al.,* (42) mention that the frequency of the molecular subtypes of breast cancers was Luminal B 139 (48.77%), Luminal A 60(21.05%), Her2 54(18.94%) and Triple Negative Breast cancer 32(11.22%). The opinion of Cho *et al.,*(27) was a pproximately 70% of breast cancers are hormonal-positive breast cancers, and they show a more favorable prognosis than HR negative breast cancers, within HR-positive/HER2-negative breast cancer, 90%-95% of tumors are luminal A and B subtypes (41,42). The current results agreed with the Arabic area study done by Almarzooq *et al.;* (2018)(39) who found that the rates were, (51.4%) luminal A, 48\147 (16.8%) luminal B subtype, 41\147 (14.3%) HER2-type and 50\147 (17.5%) patients had triple negative breast cancer.

Conclusion

Age is a well-established risk factor for breast cancer, rates of breast cancer are low in women under 40 but it increase sharply after this age. The variation of ER/PR/HER2 subtypes widen with age and race. As a result to the variation in the life style, socio-demographic characteristics, biological risk factors and different environment for the populations, all that leads to widely variation in the molecular subtype classification for breast cancer expression. Tumor presentation varies among molecular subtypes; this information may be useful in selecting treatment strategy, so the molecular classification is an essential in predicting the outcome as a prognostic tool in breast cancer.

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