Frequency of new molecular subtypes of breast cancer in Mosul

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Abstract:
Molecular subtyping of breast cancer is widely used clinically to determine the therapeutic approach and predict the prognosis; we used Immunohistochemical study (IHC) and Fluorescent *Insitu* Hybridization (FISH) as surrogates for gene expression analysis to determine the distribution of new molecular subtypes of breast cancer in mono-institution in Nineveh, using the most recent classification criteria. In this cross-sectional study, we verify the frequency of new molecular subtypes of breast cancer in 350 patients who were diagnosed as invasive breast cancer and treated in Mosul Oncology Hospital. Based on Estrogen receptor (ER), Progesterone receptor (PR), HER2 and Ki67 index, the distribution of molecular subtypes of breast cancer in our study were as follow; Luminal A (ER+/PR+/HER2-/Ki67≤20%) was observed in (38.6%), Luminal B HER2-ve subtypes (ER+/PR+/HER2-/Ki6>20%) was found in (22%), Luminal B HER2+ (ER+/PR+/HER2+) was found in (20.3%), HER2 enriched subtypes (ER-/PR-/HER2+) was observed in (7.4%) and finally Triple-negative subtypes (ER-/PR-/HER2-) was observed in (11.7%). We have found that different molecular subtypes were associated with a different clinical and pathological characteristic, additionally; this study demonstrated that molecular subtypes of breast cancer were associated with a significant difference in distal metastatic patterns in which bone metastasis was found to be the commonest site with predominance in Luminal A subtypes. In conclusion, breast cancer is heterogeneous diseases and new molecular subtyping could predict the potential anatomical site of distal metastasis and may help in tailing adjuvant therapy and surveillance protocol for breast cancer patients.

Key words: Estrogen receptor, HER2, Breast cancer, New Molecular classification, Mosul, Ki67

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Introduction

As in most of the Middle East countries [(1)], breast cancer is the most frequently diagnosed cancer among women in Nineveh province of Iraq [(2)], it remains the major cause of cancer-related mortality in Iraq and its incidence rate has gradually increased especially during the last few years, as demonstrated by Iraqi cancer registry [(3)].

The genomic atlas of breast cancer has emphasized it's heterogeneity with a wide spectrum of clinical and pathological characteristics leading to a considerable variation in the prognosis [(4)]. Recently, big attention has been focused on the molecular classification of breast cancer, four major molecular subtypes of breast cancer have been determined by a gene expression profiling which includes: luminal-A (LA), luminal-B (LB), human epidermal growth factor receptor-2-enriched (HER2), and basal subtypes[(5)]. Although multi gene assay is very elegant, predictive and prognostic, however, it still expensive and remains inaccessible for the majority of Iraqi women. The IHC based molecular classification of breast cancer is affordable, comparatively less expensive and providing both therapeutic and prognostic information and can be used as a surrogate for gene expression analysis[(5,7)].

Previous molecular classification, based on IHC of ER, PR, HER2, is still widely used to classify breast cancer patients with different risk categories and response to therapy [(8)], however, the main drawback of this molecular classification is the lack of distinction between Luminal A like (indolent, more endocrine sensitive, better prognosis) and Luminal B- like (more aggressive, less endocrine sensitive, worse prognosis) tumors, both of

them ER+ and HER2– but different behavior and prognosis, this differentiation carries important therapeutic implications, therefore, the Saint Gallen Guidelines recommend to include the assessment of Ki67, a nuclear marker of cell proliferation that is expressed in all phases of cell cycles except G0, for the distinction between Luminal B breast cancer that shows a higher Ki67 index than Luminal A[7,8].

The aim of this study is to determine the distribution of new molecular subtypes of breast cancer in Nineveh province based on the most recent cut off value of Ki-67, and also to verify whether this molecular subtyping has a clinical or pathological significance and the particular pattern of their distal spread.

Patients and methods

This cross-sectional study was performed on tissue samples from 350 patients who were diagnosed as invasive breast cancer and treated in Mosul Oncology Hospital (January 2018 to September 2019). Appropriate ethical approval for this study was obtained from the ethical committee of the College of Medicine/ Mosul University. Pertinent clinical and demographic data were obtained from clinical charts including patient's age, sex, type of surgery done, mammography, Ultrasound of the breast, MRI of breast and histopathology, CT scan of the chest and abdomen +/- pelvis or FDG PET/CT scan were performed to exclude visceral metastasis for patients with positive lymph node metastasis, staging for bone metastasis was accomplished using bone scan for patients with symptoms of bone metastasis.

The American Joint Committee on Cancer (AJCC) pathologic tumor-node-metastasis (TNM) classifica¬tion of Breast tumors were used for tumor staging [9]. The immunohistochemical staining method was done on formalin-fixed paraffin-embedded samples of tissue. Tissue sections were mounted on salinized slides. The immunostaining procedure was performed according to the manufacturer's protocols (Dako automation). The procedures included de-deparaffinization in alcohol and xylene. Followed by rehydration using the antigen retrieval solution (95°C for 40 minutes). After that, non-specific binding induced by endogenous peroxidase was quenched by applying 3% peroxide for 5 minutes. Then, Dako type primary antibodies (ER, PR, c-erbB-2 & Ki-67) were applied & incubated for 30 minutes. After that, the HRD solution was added for 30 minutes. Then stain development was accomplished by incubation with diaminobenzidine (DAB) for 10 minutes. Lastly, background staining was done using hematoxylin stain. Appropriate controls (internal & external) were added with each IHC run. The slides were reviewed by 2 qualified pathologists.

For assessment of ER & PR, the slides were examined, 100 tumor cells were selected. Then the ratio of the positive cells to the total was determined and reported as a percentage. The intensity of staining was also recorded. According to the ASCO & CAP guidelines, tumors having 1% or more invasive cancer cells staining were regarded as positive. The average intensity of the stain was also included in the report (weak, moderate or strong) [10, 11].

For HER2, according to the pattern of staining, the cases were scored 0, 1+, 2+, & 3+. Again, ASCO/CAP guidelines were used [12, 13], HER2 0/1+ was reported as negative status whereas Her2 3+score indicated positive results. Cases with scores of 2+ were considered equivocal and were subjected to FISH to confirm the HER2 status [14]. For Ki67, a total of 500 tumor cell nuclei were examined. The number of positive nuclei was calculated and the results were expressed as a percentage of positive cells [7].

Classification of molecular subtypes

Using the four biomarkers (ER, PR, HER2, and Ki67), the cases of breast cancer were classified into 5 molecular subtypes as follows: Luminal A, Luminal B (HER2-), Luminal B (HER2+), HER2 enriched and Triple negative. As shown in figure 1 [8].

Statistical analysis

Data management and statistical analysis were conducted using SPSS (Version 20; SPSS). For assessment of the significant differences between breast cancer patients with different molecular subtypes, the $\chi 2$test was used.

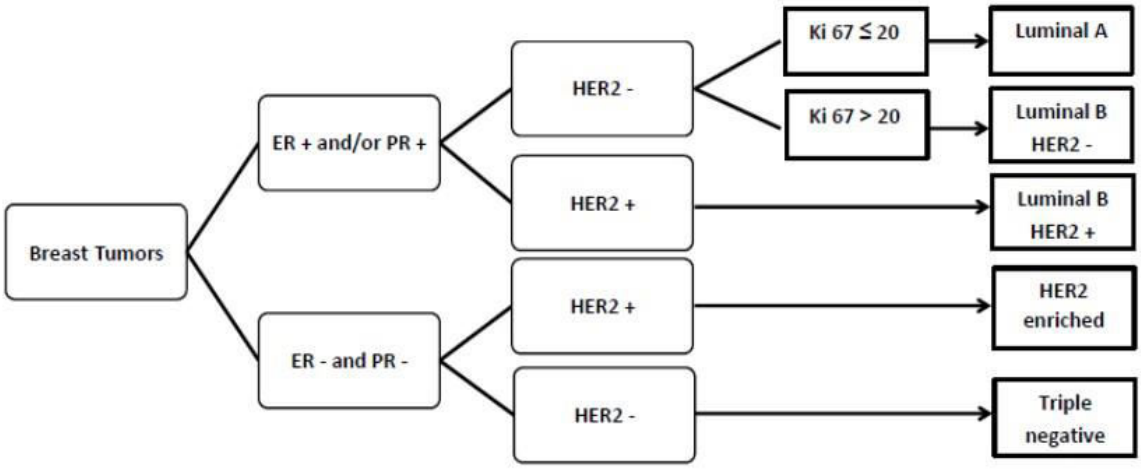

Figure 1: Classification algorithm for breast cancer molecular subtyping.

Result

This study enrolled 350 patients with invasive breast cancer patients who underwent surgical biopsies (203 Modified radical Mastectomy, 113 lumpectomies, and 34 true cut biopsy). The mean age of the patients was 50.2 years (range 21-84years). 36 patients (10.3%) were diagnosed with stage I, 140 patients (40%) were diagnosed with stage II, 133 patients (36%) were diagnosed with stage III while 41 patients (11.7%) were diagnosed with stage IV breast cancer. Out of 350 breast cancer cases, 290 (82%) were ductal carcinoma, 51 (14.6) cases were lobular carcinoma, 4 (1.1%) cases were mixed lobular-ductal, and 5 cases were others histological subtypes.

Based on immunohistochemical analysis; out of 350 cases, 281 (80.3%) cases showed positive expression of ER and 69 (19.7%) cases were negative. For HER2; 21 cases showed equivocal score and were reconfirmed by FISH which showed amplification in 11 cases, the final results were reported as follows; 97 cases (27.7%) showed positive expressions of HER2 and 253 cases (72.3%) showed negative expressions.All cases were reclassified into molecular subtypes based on immmunohistochemical analysis of ER, PR, HER2, Ki67 markers. The distribution of molecular subtypes was shown in figure 2.

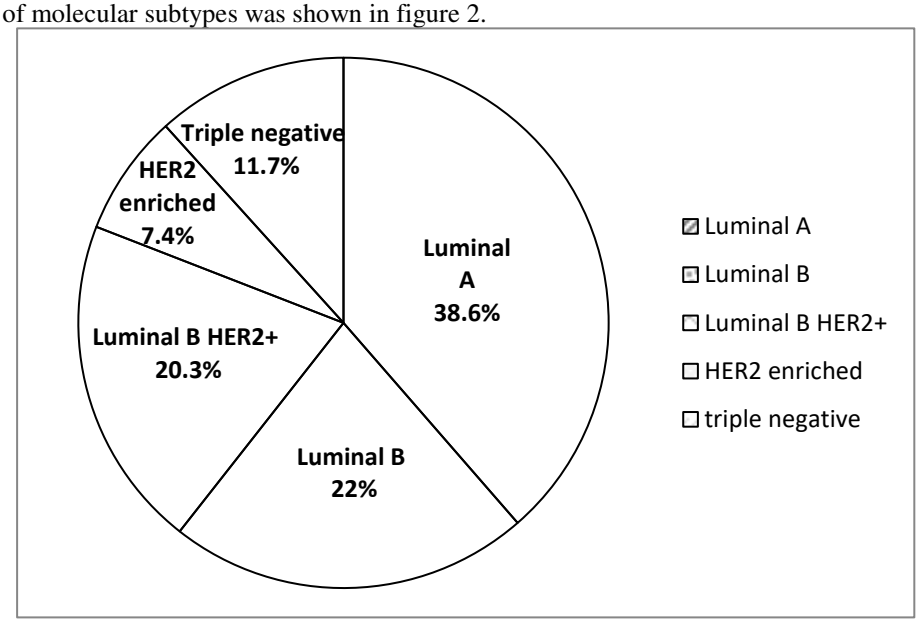

Figure 2: distribution of new molecular subtypes.

The mean age at the time of diagnosis, for patients with Luminal A, was 53.9 years (25y -84y), for Luminal B was 45.9 years (26y -77y), for Luminal B HER2 positive was 49.9 years (25y -75y), for HER2 enriched was

46.6 years (21y -65y), while for the mean age at the time of diagnosis for triple negative subtypes was 48.5 years (25y -81y). Luminal A breast cancer subtypes affected the older age group compared with other subtypes of breast cancer as shown in table 1.

Table 1: Age distribution according to molecular subtypes of breast cancer.

Age	No.	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2 enriched	Triple negative	P-value*
		No. (%)	No. (%)	No. (%)	No. (%)	No. (%)	
≤ 30	14	2 (14.3)	2 (14.3)	4 (28.6)	4 (28.6)	2 (14.3)	0.788
31 – 40	63	10 (15.9)	29 (46)	14 (22.2)	1 (1.6)	9 (14.3)	0.000
41 – 50	116	44 (37.9)	24 (20.7)	23 (19.3)	10 (8.6)	15 (12.9)	0.000
51 – 60	85	41 (48.2)	14 (16.5)	13 (15.3)	9 (10.6)	8 (9.4)	0.000
61 – 70	52	31 (59.6)	3 (5.8)	10 (19.2)	2 (3.8)	6 (11.5)	0.000
> 70	20	7 (35.0)	5 (25.0)	7 (35.0)	0 (0.0)	1 (5.0)	0.027
Total	350	135(38.6)	77(22.0)	71(20.3)	26(7.4)	41(11.7)	0.000

Table 2 showed distributions of histological subtypes, stage of the disease and grade for each molecular subtypes. A significant association was found between the molecular subtypes of breast cancer with the stage of the disease (p value=0.000), HER2 enriched and Triple-negative subtypes have higher percentages of advanced stage compared with Luminal A and Luminal B breast cancer subtypes. Furthermore, the most significant parameter in the staging was lymph node status, (p value=0.006) in which the amount of lymph node metastasis was found among patients with HER2 enriched and Triple-negative subtypes compared with other subtypes of patients. Whereas, no significant association between the pattern of expression of ER, PR, and HER2, and the histopathological types and the tumor size (Table 2). Luminal A and Luminal B subtypes comprised higher proportions of Grade I and Grade II tumors, while HER2 positive and triple-negative subtypes comprised a higher proportion of Grade III tumors, as seen in table 2.

Table 2: Correlation of molecular subtypes withclinicopathological features of 350 cases of breast cancer.

	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2 enriched	Triple negative	Total	P-value
Number(%)	135 (38.6%)	77 (22%)	71 (20.3%)	26 (7.4%)	41 (11.7%)	350 (100%)	
Stage							
stage I	20	4	5	2	5	36	0.000
stage II	72	21	26	7	14	140	
stage III	37	36	33	11	16	133	
stage IV	6	16	7	6	6	41	
Total	135	77	71	26	41	350	
Tumor							
T1=>2cm	23	6	14	2	4	49	0.05
T2=2-<5cm	91	55	51	18	29	244	
T3=>5cm	18	15	6	3	6	48	
T4	3	1	0	3	2	9	
Total	135	77	71	26	41	350	
Nodes							
N 0	48	13	12	3	10	86	0.006
N1	43	20	19	5	10	97	
N2	28	30	26	9	12	105	
N3	7	10	11	7	5	40	
NX	9	4	3	2	4	22	
Total	135	77	71	26	41	350	
Grade							
grade I	7	0	0	0	0	7	0.000
grage II	66	20	10	5	5	106	
grade III	62	57	61	21	36	237	
Total	135	77	71	26	41	350	

Histology							
Ductal	111	62	61	25	31	290	0.173
Lobular	22	11	9	1	8	51	
Mixed	0	3	1	0	0	4	
others	2	1	0	0	2	5	
Total	135	77	71	26	41	350	

In our study, 41 patients were diagnosed as stage IV diseases, which account for (11.7%) of all patients, of whom, 18 patients (43.9%) presented with multiple metastatic sites, and 23 patients presented with only one site of metastasis, as seen in figure (3).

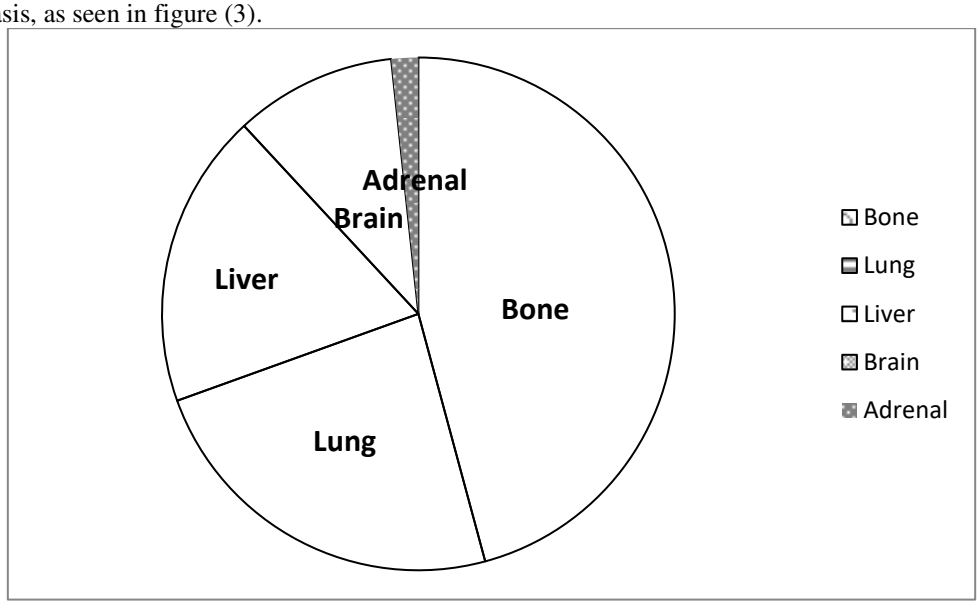

Figure 3: frequency of distal metastasis.

Different frequencies of the site of distant metastasis were found among different breast cancer subtypes. Bone metastasis was most frequent in all subtypes with significant predominance in the Luminal A group, brain metastasis was more frequently observed in HER2 enriched subtypes, while liver metastasis was frequently observed in triple negative subtypes, as shown in table 3.

Table 3: Site of metastasis in relation with new molecular subtypes of breast cancer.

Site of metastasis	No. = 41	Luminal A	Luminal B (HER2-)	Luminal B (HER2+)	HER2 enriched	Triple negative
Multiple sites	18	4	9	2	1	2
Only one site	23	2	7	5	5	4
Bone	27	6	15	4	2	0
Lung	14	3	7	2	0	3
Brain	6	0	0	2	4	0
Liver	11	1	3	2	0	5
Adrenal	1	0	0	0	1	0

Discussion

Molecular subtyping of breast cancer is widely used clinically to determine the therapeutic approach and predict the prognosis[15], however, such subtyping has not been practiced in Nineveh province previously and this is the first attempt for such classification. We have used IHC and FISH as surrogates for gene expression analysis to determine the distribution of new molecular subtypes of breast cancer in mono-institution in Nineveh.

Different cut-off values of Ki67 have been used for differentiating Luminal A from Luminal B subtypes of breast cancer, at first, Ki67 index >14% was established by the Saint Galleon consensus meeting in

2011[16],Later on, refinement of this classification was done by the Saint Gallen conference in 2013 which recommended to use a threshold of Ki67>20% for defining Luminal B subtypes [7], however, low reproducibility of Ki67 result was reported by many studies [17], thus, during the last Saint Gallen conference, the panel of experts accepted the use of a Ki67 index less than the average for ER+/HER2- tumors in the local laboratory[16].

In this study, we used Ki67 >20% as a cut off value for defining Luminal B HER2 subtypes. The frequency of different molecular subtypes of breast cancer is comparable to that of other studies [18, 19, 20, 21] although, some difference in the frequency of Luminal A and Luminal B subtypes was noted. This could be attributed to low Ki67% cut values that used for differentiating these subtypes of breast cancer in their studies, in addition to the difference in the population age structures of these countries [22] and secular increase in the incidences of Luminal A and Luminal B (HER2-) subtypes in some counties [20].

In this study, the mean age of breast cancer patients at the time of diagnosis was 50 years which was more or less similar to the average age that has been shown by other studies which were done in Iraq and other Arabic counties [23, 24], but it is much lower than the average age of breast cancer women at the time of diagnosis in the USA (>60 years) [25], this difference could be explained by the several-folds increase in the incidence of breast cancer in post-menopausal women in the older population of Western countries compared with a younger population and shorter life expectancy in Iraq and other Arabic countries [23].

Age-specific frequency of breast cancer subtypes in this study is similar to the result of the previous study that based on PAM50 molecular testing which showed that Luminal A subtypes exceed Luminal B subtypes at age > 60 years and luminal B breast cancers were found to be more common than luminal A tumors by a factor of 2.48 under 40 years of age with the ratio decreasing to 1.27 at 40–49 years old [26, 27]. On the other hand, we found that triple-negative subtypes have occurred more common in young women, this result is consistent with others which demonstrated that the triple-negative and HER2 subtype were more frequent in the young age group of Canadian, African-American and Asian patients [28, 29].

However, The distribution of breast cancer subtypes in this study slightly differs from that shown by a previous study conducted in the north of Iraq [15], which showed that Luminal A subtypes forming only 27% of breast cancer cases, and also showed that luminal B tumors were the predominant subtype at all ages, and both luminal A and luminal B tumors were unimodal and closely overlapping with no suggestion of a late peak for Luminal A tumor. This differences could be related to their study population as they include Arabic and Kurdish breast cancer women [15].

As expected, the result of the current study demonstrated that ductal carcinoma was the most frequent histological subtypes (82% of the cases), followed by lobular carcinoma (14.6% of cases). As for the grade, all grade I tumors was Luminal A subtype which reflects the favorable prognosis of this subtype, while the majority of HER2 enriched and triple-negative subtypes were of higher grade which correlates with the aggressive nature of this subtypes [21].

The association between molecular subtypes and the site of distal metastasis is not well known [5]. A better understanding of the mode of metastasis of each molecular subtypes of breast cancer may help in monitoring decisions and determining which screening and follow up strategies are appropriate for each case of breast cancer, therefore, we have verified the frequency of different sites of distal metastasis in different molecular subtypes of breast cancer who diagnosed with stage IV diseases. We found that patients with triple-negative and HER2+ breast cancer have a high prevalence of distal metastasis (14.6%) compared with Luminal A (4.4%), we also demonstrated that bone is the comment site of distal metastasis followed by lung, liver and brain metastasis. This result is inconsistent with the result of a large comprehensive study which analyzed the relationships between the pattern of distal metastasis and molecular subtyping of breast cancer [30].

HER2+ enriched subtypes and Luminal B HER2+ exhibited more metastasis to the brain than the other molecular subtypes. These results are similar to that of other studies [5,31,19,32,33] which demonstrated that HER2-positive breast cancer has a potential affinity for brain tissue, this strong relationship with brain metastasis and HER2-positive breast cancer is inconsistent with the result of another study which demonstrated that tumor which overexpressed HER2 gene metastasize three times more often to the brain [33] and there was increasing in the outgrowth of HER2 overexpressed metastatic breast tumor cells in the brain in vivo [34].

On the other hand, our study demonstrated that bone metastasis is more frequent in Luminal A subtype, similar to the result of others, which demonstrated that bone was the commonest site of distant metastasis especially in Luminal A and B[5], which may be attributed to the strong association between ER and bone metastasis in breast

cancer [35]. Furthermore, Caldeira P; et al found that about 70% of metastatic bone Luminal A breast cancers were found in flat bone and this could be very beneficial in clinical practice of oncologist.

In conclusion, breast cancer is heterogeneous diseases and molecular subtypes are associated with a different clinical and pathological characteristic, additionally, this study demonstrated that molecular subtypes of breast cancer were associated with a significant difference in distal metastatic pattern, bone metastasis was found to be the commonest site with predominance in Luminal A subtypes. So this molecular subtyping could predict the potential anatomical site of distal metastasis and may help in tailing adjuvant therapy and surveillance protocol for breast cancer patients.

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