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Original Article

Molecular subtyping of breast cancer patients with long time follow up and its prognostic value on survival: a single center analysis

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Abstract: The importance of molecular subtyping in breast cancer is an unresolved issue. In this study we aimed to evaluate the significance of molecular subtyping, and the correlation between the disease-free, and overall survival in breast cancer based on molecular subtypes. A total of 536 patients with the diagnosis of breast cancer between the years 1980 and 2014 were included in the study. Tumors were divided into five molecular subtypes according to their expression profiles as follows: Luminal A: (n=220; 41%); Luminal B: (n=72; 13.4%); Luminal B-like: (n=97, 18.1%); HER2: (n=44;8.2%); and Triple-negative (n=103;19.2%). We found significant differences between molecular subtypes, and histological subtype of the tumor (P=0.004) in terms of local recurrence (P=0.043), and metastasis (P=0.006). A statistically significant difference was found between the number of metastases, and molecular subgroups. (P=0.037). Among all molecular subtypes, local recurrences (11.4%), and metastasis (38.6%) were most frequently seen in the HER2 subtype, while the least number of metastases (15.3%) were detected in the Luminal A subtype. A statistically significant difference was found between Luminal A, and HER2 subgroups as for incidence of metastatic lesions (P=0.007). However in the Luminal A subgroup metastases developed in the long term (at the end of 50 months after onset of the disease). Overall, and disease-free survival curves in the Luminal A subgroup indicated risk of mortality in the long run. Based on molecular subtyping the worst, and the most favourable survival rates were observed in the HER2, and Luminal A subgroups, respectively. Impact: In this study which encompassed multiple number of breast cancer patients encountered within 30 years, HER2 tumors had the worst survival rates Interestingly, Luminal A subgroup which displayed a very favourable prognosis during the early stage of the follow-up period, demonstrated a bad prognosis in the long term.

Keywords: Breast cancer, molecular subtype, prognosis, survival

Introduction

Breast cancer is one of the most prevalently seen malignant tumors among women in the world. Efficient use of routine screening methods, and imaging modalities, increase in the opportunities of early diagnosis, common, and appropriate use of treatment protocols, discovery of target treatment facilities have decreased mortality rates. Still an increase in the incidence of breast cancer is seen.

Breast cancer is known to demonstrate variations in many ways including its clinical presentation, biological behaviour, and treatment response. Traditional histological classification fails to meet this multifaceted heterogeneity. Therefore nowadays, breast cancer researches have changed from histological to molecular classification. In the year 2000, a Californian group of researchers suggested molecular classification system based on gene expression pattern in breast cancer which defined four subgroups [1]. In more recent studies performed by different breast cancer study groups, these defined groups were confirmed, and received global acceptance.

In the St. Gallen International Expert Consensus Conference organized in 2011, a new classification system where breast cancer was divided into five different subgroups was suggested. In

the year 2013, criteria used to define these five different subgroups were defined clearly. Based on these criteria the subgroups were classified as follows: 1- Luminal A (ER positive, HER2 negative, lower Ki67 proliferation index, and higher PR positivity), 2- Luminal B (HER2 negative): ER positive, HER2 negative, and higher Ki67 proliferation index or lower PR, 3- Luminal B-like (HER2 positive): ER positive, HER2 positive (overexpressed or amplified), Ki67, and PR at any state, 4- HER2 positive: HER2 overexpressed or amplified, ER and PR negative, 5- Triple-negative: ER, PR, and HER2 negative. These molecular groups correlate with biochemical biomarkers. In new studies, differences in response to treatment, and course of the disease have been demonstrated.

In this study we aimed to investigate whether molecular subgrouping in breast cancer is superior over conventional histopathological evaluation. We also intended to evaluate the correlation between predefined molecular subgroups of breast cancer as for event-free survival (EFS), and overall survival (OS). A cohort containing 536 breast cancer patient groups with a survival time of 30 years was re-classified according to molecular subtypes. In this subtyping, widely used biomarkers as oestrogen receptor (ER), progesterone receptor (PgR), HER2, and Ki67 were utilized.

Material and method

The patients treated, and followed-up with the diagnosis of breast cancer between 1980, and 2014 years in Izmir-Bozyaka Training and Research Hospital were included in the study. The patients were treated with modifed radical mastectomy, lumpectomy, breast-sparing surgery, and one of the surgical interventions recommended within the years where cases of cancer were detected. Later on clinicopathological evaluation was made, then in line with the decisions of the council, chemotherapy, radiotherapy, and the appropriate treatment selected among target treatment programs valid during the years of diagnosis were applied. Electronic database related to the the time period extending from 1980 up to September 2014 contained information about a total of 768 patients. The patients whose hormone receptors (n=54), and cerbB2 protein (n=66) expressions, and Ki67 proliferation indices (n=112) were not evaluated or recorded were excluded from the study leaving 536 patients as study participants.

Histopathological, immunohistochemical evaluations

Histopathological, and immunohistopathological diagnoses were based on the assessments of pathologists working in the pathology laboratory, and a senior pathologist was consulted for each pathology slide. Clinical, and histopathological data were retrieved from archival files. Local recurrence was accepted as any localized lesion in the breast tissue, mastectomy scar, ipsilateral axillary lymph nodes. Distant metastasis was defined as any localized lesion other than breast tissue, mastectomy scar, ipsilateral axillary and/or supraclavicular lymph nodes. Cases with bilateral breast cancers were included in the study. A time interval of 3 months was accepted as a criterion used to make a differentiation between metachronous, and synchronous bilateral breast cancers.

ER, and PgR positivity were determined using immunohistochemical. assessments, and all data were retrieved, and recorded from electronic media screening. Staining of more than 1% of tumor cells was accepted as ER or PgR positivity. Ki-67 proliferation index was also determined based on the results of immunohistochemical evaluation. Nuclear staining detected in more than 15% of tumor cells was accepted as a criterion of positivity. HER2-receptor state was determined based on immunohistochemically evaluated archival data. IHC 2+ tumor specimens were also evaluated using florescence in-situ hybridization (FISH) method with signal amplification. Apart from IHC-negative (score 0, and 1) or positive (score 3) cases, HER2-receptor state of each case was determined based on the FISH results.

All patients were divided into subgroups based on the above-defined evaluation criteria as Luminal A (ER and/or PgR positive, HER2 negative, Ki-67 proliferation index <15%), Luminal B (ER and/or PgR positive, HER2 negative, Ki-67 proliferation index >15%), Luminal B-like (ER and/or PgR positive, HER2 positive, any Ki-67 proliferation index), HER positive (ER, and PgR negative, HER2 positive) and triple-negative (ER, PgR, and HER negative).

Table 1. Descriptive statics for the 536 breast cancer cases

	Luminal A	Luminal B	Luminal B-like	HER2	Triple negative	Total
Number (%)	220 (41)	72 (13.4)	97 (18.1)	44 (8.2)	103 (19.2)	536
Mean age (SD)	55 (13.4)	51.9 (13.4)	52.8 (12.2)	51.9 (10.7)	49.9 (12.8)	53.3 (13)
Menopausal status						
Premenopausal	72 (32.9)	34 (47.2)	38 (39.6)	20 (45.5)	57 (55.3)	221 (41.2)
Postmenopausal	145 (66.2)	38 (52.8)	57 (59.4)	23 (52.3)	45 (43.7)	308 (57.7)
Laterality						
Unilaterality	203 (92.3)	69 (95.8)	88 (90.7)	41 (93.2)	99 (96.1)	500 (93.3)
Bilaterality	17 (7.7)	3 (4.2)	41 (93.2)	3 (6.8)	4 (39.0)	36 (6.7)
Tumor type						
IDC	157 (72)	58 (80.6)	75 (77.3)	39 (88.6)	61 (59.8)	390 (73.2)
ILC	30 (13.8)	8 (11.1)	7 (7.2)	1 (2.3)	15 (14.7)	61 (11.4)
IDC+ILC	14 (6.4)	3 (4.2)	9 (9.3)	2 (4.5)	8 (7.8)	36 (6.8)
Mucinous	11 (5.0)	1 (1.4)	3 (3.1)	-	1 (1.0)	16 (3)
Medullary	-	-	1 (1.0)	-	9 (8.8)	10 (1.9)
Tubular	3 (1.4)	-	-	-	1 (1)	4 (0.7)
Cribriform	1 (0.5)	1 (1.4)	-	-	-	2 (0.4)
Papillary		1 (1.4)	-	-	1 (1)	2 (0.4)
Other	2 (0.9)	-	2 (2.1)	2 (4.5)	6 (5.9)	12 (2.3)
Tumor size[a] n (%)						
T1	67 (30.5)	15 (20.8)	20 (20.6)	9 (20.5)	29 (28.2)	140 (26.2)
T2	103 (46.8)	37 (51.4)	58 (59.8)	21 (47.7)	52 (50.5)	271 (50.7)
T3	20 (9.1)	10 (13.9)	6 (6.2)	7 (15.9)	8 (7.8)	51 (9.5)
T4	19 (8.6)	5 (6.9)	6 (6.2)	3 (6.8)	5 (4.9)	38 (7.1)
LN status[a]						
N0	86 (39.3)	23 (31.9)	33 (34.0)	14 (31.8)	48 (46.6)	204 (38.1)
N1	56 (26.6)	15 (20.8)	25 (25.8)	9 (20.5)	24 (23.3)	129 (24.1)
N2	41 (18.7	23 (31.9)	20 (20.6)	9 (20.5)	12 (11.7)	105 (19.6)
N3	26 (4.6)	11 (15.3	16 (16.5)	11 (2.5)	15 (14.6)	79 (14.8)
Stage[b]						
I	46 (21.0)	5 (6.9)	12 (12.4)	3 (6.8)	20 (19.4)	86 (16.1)
II	88 (40.2)	26 (36.1)	41 (42.3)	18 (40.9)	45 (43.7)	218 (40.7)
III	69 (31.5)	35 (48.6)	39 (40.2)	21 (47.7)	28 (27.2)	192 (35.9)
IV	8 (3.7)	2 (2.8)	1 (1.0)	-	1 (1.0)	12 (2.2)
Unknown	8 (3.7)	4 (5.6)	4 (4.1)	2 (4.5)	8 (3.7)	27 (5.0)
Local nux	5 (2.3)	1 (1.4)	5 (5.2)	5(11.4)	4 (3.9)	20 (3.7)
Metastasis	33 (15.3)	13 (18.3)	25 (25.8)	17 (38.6)	20 (19.6)	108 (20.1)
Metastasis type						
Single	27 (12.5)	8 (11.3)	20 (20.8)	9 (20.5)	16 (15.5)	80 (14.9)
Multipl	15 (6.9)	7 (9.9)	9 (9.4)	8 (18.2)	7 (6.9)	46 (8.6)

[a]Histologically confirmed; [b]Combined clinical and histological stage.

Statistical analysis

Continuous data were expressed as means ± standard deviations (SD) and categorical data as frequencies, counts and percentages. One-Way ANOVA test was used to evaluate differences between molecular subtypes. *P* values were considered significant if less than 0.05. Event free survival (EFS) was considered as the time interval between the time of the diagnosis, and detection of local recurrence and/or metastasis. Overall survival (OS) was estimated

Table 2. Comparisons between molecular subgroups

Comparison of Subtype	Tumor Type	Local nux	Metastasis	Metastasis Type Single/Multipl
Luminal A x Luminal B	1.216	*0.048*	0.069	0.114
Luminal A x Luminal B-like		*0.014*	*0.012*	*0.014*
Luminal A x HER2	1.412	*0.029*	0.108	0.103
Luminal A x Triple negative	0.265	*0.023*	*0.045*	0.107
Luminal B x Luminal B-like	0.679	*0.020*	0.055	0.116
Luminal B x HER2	1.080	*0.016*	*0.039*	*0.009*
Luminal B x Triple negative	0.466	*0.026*	0.108	0.203
Luminal B-like x HER2	0.906	*0.030*	*0.035*	0.080
Luminal B-like x Triple negative	0.578	0.071	0.179	0.276
HER2 x Triple negative	0.271	0.160	0.343	0.508

from the date of cancer diagnosis to the date of death from any cause. The impact of subtypes on EFS and OS was assessed by means of Kaplan-Meier test. SPSS 18.0 sofware was used for statistical analysis.

Results

Distinctive clinicopathological features

A total of 536 cases (male, n=5, 0.5%; and female, n=531, 99.5%) with invasive breast carcinoma were included in the study. Two hundred and twenty-one patients (41.4%) were in their premenopausal period. Mean age of the patients was 52.3 years (23-84 years). Involvement of the right (n=236, 44%), left (n=264; 49.3%), and both (n=36; 6.7%) breasts was detected in respective number of patients. Most widely encountered morphological category was invasive ductal carcinoma (n=390; 73.2%) Other types, included lobular carcinoma, mixed carcinoma, cribriform carcinoma, papillary carcinoma and mucinous carcinoma. Mean tumor size was 3.2 cm (range 0.1-14 cm). In 204 (38.1%) patients lymph node metastasis was not found. In most of the patients (n=184; 34.3%) more than 3 metastatic lymph nodes were detected. The patients were followed up for an average period of 81 months (0.4-401.3 mos).

Distribution of molecular subtypes

The distribution of subtypes was as follows: Luminal A (n=220; 41%); Luminal B (n=72 cases; 13.4%), Luminal B-like (n=97; 18.1%); HER2 (n=44; 8.2%); Triple negative (n=103; 19.2%). All clinicopathological characteristics of the patients according to molecular subtypes are given separately in **Table 1**.

One-Way ANOVA test was used to evaluate differences between molecular subgroups with respect to histopathological subtype, TNM stage, and local recurrences of the breast cancer. We detected significant differences among molecular subtypes, histopathological subtypes (P=0.004), local recurrences (P=0.043), and metastases (P=0.006). Since, priorly we wanted to evaluate the correlation between molecular subtyping, and prognosis in breast cancer, the number of metastases (single or multiple) was also assessed. A statistically significant difference was detected between molecular subgroups regarding the number of metastases (P=0.037).

Student-T test for independent variables was performed to determine which subtypes were responsible for this difference. Comparisons between molecular subgroups are seen in **Table 2**.

Among all molecular subtypes local recurrence was seen most frequently (11.4%) in HER2 subgroup. Local recurrences were seen in decreasing order of frequency as follows: Luminal B-like (5.2%), Triple-negative (3.9%), Luminal A (2.3%), and Luminal B 1.4%. Similarly, metastases were most frequently (38.6%) seen in the HER2 subgroup followed by Luminal B-like (25.8%), Triple-negative (19.6%), Luminal B (18.3%), and Luminal A (15.3%).

Mean occurrence of metastases in all molecular subgroups after diagnosis of breast cancer varied widely (HER2, 25.6±20.41 mos; Luminal B-like, 35.0±26.7 mos; Triple-negative, 46.5±30.0 mos; Luminal B, 47.8±29.7 mos; Luminal A, 50.3±41.6 mos). Frequency of metatases, and shorter interval from the time of diagnosis up to the development of metastases correlated between subgroups. A statistically significant correlation was detected between groups as for the time elapsed from the diagnosis of the disease up the occurrence of metastases

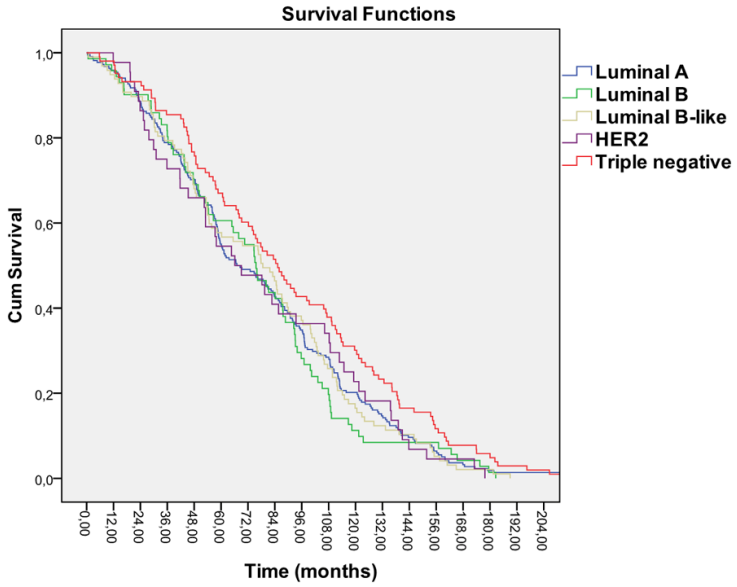

Figure 1. Kaplan-Meier survival curve for overall survival.

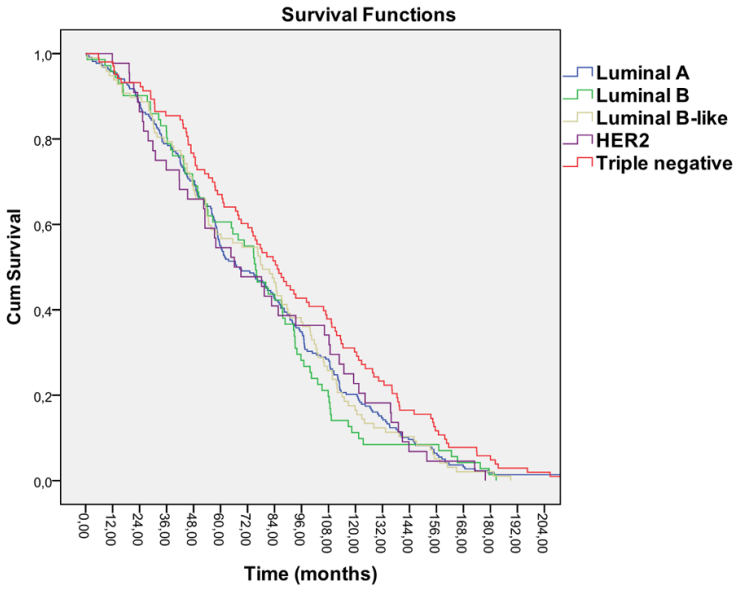

Figure 2. Kaplan-Meier survival for event free survival.

as evaluated using one-way ANOVA test (P= 0.081). Statistically significant differences (if any) between groups were investigated using independent Student's T test. A statistically significant difference was detected between Luminal A, and HER2 subgroups as for the time to the occurrence of metastases (P=0.007).

We encountered local recurrences, and metastatic lesions after a long term in patients with Luminal A subtype breast cancer (at the end of 75, and 50 months after onset of the disease, respectively). Kaplan-Meier survival analysis was performed between subgroups, and log-rank test did not reveal any statistically significant differences between subgroups (P=0.159) (**Figures 1** and **2**).

Discussion

The decision of adjuvant systemic treatment changes according to various parametres including tumor size, nodal involvement, ER, PgR, and HER status, and Ki-67 proliferation index. Therefore national, and international guidelines suggest the necessity of including these parametres in pathology reports [2, 3]. However in order to facilitate the comprehension of complex heterogeneity, and prognostic factors related to breast cancer, in recent years various studies have been performed on gene expression profiles. Despite all of these efforts, definitions of molecular subtypes of breast cancer have not been standardized so far. Scarce number of prospective studies which can validate distribution of subtypes in addition to clinicopathological parametres have been performed so far [4, 5]. Gene expression profiling does not replace traditional histopathological evaluation, and it is used as a complementary method in cases where biological behaviour of the tumor is included in histopathological evaluation [6].

In the present study, we priorly evaluated frequency of molecular subtypes in a cohort consisting of numerous, and widely distributed breast cancer patients with longer follow-up who were treated in a single center in Turkey. We based our assessments on widely used, and precisely defined immunohistochemical

evaluation criteria related to ER, PgR, HER2, and Ki67. The most prevalently seen breast cancer type was Luminal A subtype (41%). Various reports coming from many regions of the world have indicated Luminal A subtype as the most prevalently seen type [7-11]. Discrimination between Luminal A and Luminal B tumor subtypes is made based on the cut-off point of Ki67 proliferation index which is a complex, and problematic method. In this study we determined a cut-off value of 15% for Ki67 proliferation index in line with currently valid recommendations of St Gallen International Expert Consensus [3]. However, since Ki-67 is a continuous variable, increasing its cut-off value to 20% was discussed critically in 13th St Gallen Consensus Conference. At present cut-off value for Kİ67 has not been standardized yet. Therefore relatively limited number of studies have been compared based on Ki67 proliferation indices so far [12].

As reported in many studies, in our study Luminal B was the second most prevalently seen subtype [13, 14]. In many studies the incidence of triple negative-basal like subtype has been reported to vary between 15, and 20 percent [11, 15-18]. We also obtained similar incidence rate (19.2%). However in some other studies very high incidence rates have been reported [19, 20]. Diverse incidence rates have been also reported for HER2 subtype. Our incidence rates for HER2 subtype were also in consistent with the outcomes of the studies performed separately by Cherbal et al. and Zheng et al. [11, 20]. In various studies very high or very low incidence rates have been reported [15, 21]. Widely different range of values detected for HER2, and triple negative subtypes may be attributed to different genetic backgrounds, geographic factors, and etiological heterogeneity.

In this study in addition to classifying breast cancer patients into 4 molecular subgroups, we also evaluated correlations between histopathological subtype, TNM stage, local recurrence and metastasis between these subgroups. Since we detected correlations between molecular subgroups as for local recurrence, and metastasis, after this part of the study we analyzed the difference between disease-free, and overall survival rates. As can be expected, Luminal A has the most favourable disease-free survival, followed by Luminal B. Elizabeth et al. also found similar outcomes [14]. HER2 subtype had the worst prognosis among all subtypes. In some studies, basal-like subgroup was also included in the molecular subgrouping. In these studies HER2, and basal-like subgroup had the worst prognosis [6, 8, 14].

More favourable prognosis in Luminal B- like subtype, when compared with, HER2 subtype was explained by its hormone-receptor positivity. Engstrom et al. also demonstrated that Luminal B-like subgroup has better prognosis than, HER2 subgroup [8]. This phenomenon demonstrates the importance of hormone receptor state in prognosis of breast cancer. In some studies, HER2-positive patients were not further subclassified into Luminal B-like subgroup before detailed evaluation [14]. They indicated that since HER2 positivity is a marker which is completely unrelated to hormone-receptor state, further subclassification into Luminal B-like subgroup is not necessary.

Although a significant correlation exists between subgroups of HER2, and Luminal A as for the presence of metastases (P=0.007, Student t test), insignificant result obtained in the Kaplan-Meier survival analysis, and log-rank test can be explained by very long follow-up period, and development of metastases at a very late stage in the Luminal A subgroup. Though Haque et al. demonstrated that Luminal A subtype has the optimal prognosis, they also emphasized late-term mortality detected in this subtype after 10 years of follow-up which was similar to our outcome [22]. In a study performed on the relationship between molecular subgroup, and survival, Luminal A was detected as the subgroup with the most favourable prognosis [14]. However in this study patients were followed up for only 7 years. Besides in this study Luminal B-like group was not defined.

Certain limitations of this study should be also taken into account. Since we performed molecular subtyping using immunohistochemical markers, some patients may be misclassified. In some studies immunohistochemical, and gene expression profiles have been used for subtyping [23-25]. Another limitation is that patient group might receive inadequate treatment before introduction of trastuzumab into clinical use in our country. However we think that since we investigated patient series with

greater number of patients, this erroneous grouping, and the number of inadequately treated patients did not effect statistical evaluations adversely.

In summary, our results represent numerous breast cancer patients during a long-term observation period. Most of the studies on survival rates have evaluated a period of 5-10 years, we performed molecular subtyping in a patient group which we followed up for more than 20 years. We detected many findings compliant with the literature. Luminal A subgroup which displayed a good prognosis in the early stages of the follow-up period, while as an interesting observation in the long-term it had a bad prognosis. Future studies should take long-term follow-up data covering more than 10 years, and different population analysis criteria as race, and geographic etiologies into consideration when investigating the correlation between molecular subtyping, and survival rates.

Disclosure of conflict of interest

None.

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