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Presentation of the Molecular Subtypes of Breast Cancer Detected By Immunohistochemistry in Surgically Treated Patients

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Abstract

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INTRODUCTION: The detection of estrogen, progesterone and HER-2 neu receptors on the surface of the tumour cell is a significant prognostic factor, alone or in combination. The presence or absence of receptors on the surface of the tumour cell is associated with the conditional gene expression in the tumour cell itself. Based on these genetically determined expressions of the tumour cell, five molecular subtypes of breast cancer have been classified on the St. Gallen International Expert Consensus in 2011 that can be immunohistochemically detected, with each subtype manifesting certain prognosis and aggression.

AIM: Analyzing the presentation of molecular subtypes of breast cancer that are immunohistochemically detected in surgically treated patients at the Clinic for Thoracic and Vascular Surgery.

MATERIAL AND METHODS: We used the international classification on molecular subtypes of breast cancer which divides them into: Luminal A (ER+ and/or PR+, HER-2 negative, Ki-67 < 14%), Luminal B with HER-2 negative (ER+ and/or PR+, HER-2 negative, Ki-67 ≥ 14%), Luminal B with HER-2 positive (ER+ and/or PR+, HER-2+, any Ki-67), HER-2 enriched (ER-, PR-, HER-2+), and basal-like (triple negative) (ER-, PR-, HER-2 negative, CK5/6+ and/or EGFR+). A total of 290 patients, surgically treated for breast cancer, were analysed during 2014.

RESULTS: In our analysis, we found that Luminal A was present in 77 (26.55%) patients, Luminal B HER-2 negative was present in 91 (31.38%) patients, Luminal B HER-2 positive was present in 70 (24.14%) patients, HER-2 enriched was present in 25 (8.62%) patients and basal-like (or triple negative) was present in 27 (9.31%) patients.

CONCLUSION: Detecting the subtype of breast cancer is important for evaluating the prognosis of the disease, but also for determining and providing an adequate therapy. Therefore, determining the subtype of breast cancer is necessary for the routine histopathological assay.

Introduction

Breast cancer is the most frequent malignant disease among women worldwide, but also in the Republic of Macedonia [1] [2] [3] [4]. Also, breast cancer is the leading cause of cancer mortality [1] [2] [3] [4]. Today, many factors are listed as most

important ones for determining the prognosis of breast cancer, like tumour size, histologic subtype, tumour grade, lymphovascular invasion of tumour cells and axillary lymph node status. However, the presence of hormonal receptors (estrogen and progesterone) on the surface of the tumour cell, the presence of HER-2 neu receptor and other factors have been added to this list in the last twenty years [5]. Also, the biological

potential for proliferation and dividing was routinely examined, represented as Ki67 value. Today, every patient that is surgically treated undergoes routine examination with standard macroscopic and microscopic histological analysis, TNM staging, staging by immunohistochemical recognition of estrogen receptors (ER), progesterone receptors (PR), the presence of HER-2 neu receptors, and the prognostic value of Ki67 [6]. Each of these parameters, alone or in combination, determines the biology and aggression of the tumour, but also gives us the opportunity to treat the given type of breast cancer properly. The combination of these parameters gives us the opportunity to determine the genetic subtype of breast cancer. According to the new classification system for breast cancer subtypes presented in St. Gallen, which we use, breast cancer is divided in Luminal A, Luminal B with HER2 negative, Luminal B with HER2 positive, HER2 enriched and basal-like (triple negative) [7].

Analyzing the presence of breast cancer subtypes in our materials, and comparing the results with other studies to see if some subtypes in our materials differ from other studies published before, also, determining if the subtypes and the clinical stage are somehow correlated.

Material and Methods

A total of 290 patients, who were surgically treated for breast cancer at the University Clinic for Thoracic Surgery, Skopje, Republic of Macedonia were analysed during 2014, with complete history, using all parameters.

All cases underwent standard histological examination, including macroscopic and microscopic analysis with standard H&E staining. Immunohistochemical staining for ER, PgR, HER-2 and Ki-67 were performed on sections of formalin-fixed paraffin-embedded tissue from the primary tumours. Pathohistological tests were conducted in three accredited laboratories (two in the Institute of Pathology at the Medical Faculty in Skopje and one in a private laboratory).

Upon microwave-pretreated in citric acid (10 mM), monoclonal mouse antibody to ER, PgR, HER-2 or Ki-67 was applied for 30 min at room temperature using the following dilutions: anti-ER-1 : 100, anti-PgR-1 : 80 (DAKO laboratories, UK); pre-diluted anti-HER-2 (Hercept test, DAKO Laboratories, UK); anti-Ki-67-1 : 200 (DAKO Laboratories, UK). Upon three rinses in Tris-buffered saline (TBS) and incubation with the secondary antibody, positive brown staining was detected using standard avidin and biotinylated horseradish peroxidase (ABC) technique with 3, 3'-diaminobenzidine (DAB) as the

chromogen. Slides were then counterstained in Mayer's haematoxylin for 10 seconds, dehydrated in graded alcohol, mounted and scored.

Positive and negative controls were performed with each stain, and surgical specimens from the same patient were stained on the same run.

For persistence of estrogen and progesterone receptors were included all results with +, ++ or +++ on immunohistochemical examination. For persistence of HER-2 receptors were included all patients with +++ result on immunohistochemical analysis.

In cases where ICT determined HER-2 neu positive status + or ++ patients underwent FISH analyses for defining the HER2-neu gene amplification status.

Pathohistological, grading and staging criteria for breast cancer were determined by using criteria from American Joint Committee (AJC) and TNM classification according to UICC (International Union for Cancer Control) [8] [9]. According to the new classification system for breast cancer subtypes presented in St. Gallen, which we use, breast cancer is divided in Luminal A, Luminal B with HER2 negative, Luminal B with HER2 positive, HER2 enriched and basal-like (triple negative) [7].

Statistical analysis was performed with Statistica 7 by using standard descriptive analyses, χ^2 test and ANOVA test for analysing the variance.

Results

Patient's age was ranged between 18-90 years, an average of 57.6 years. The mean size of a primary tumour was 30.27 ± 18.3 mm. Axillary lymph nodes metastases were detected in 59% of the patients.

We used the new St. Gallen classification system for defining breast cancer subtypes into five groups. (Table 1) [7].

Table 1: Definition of subtypes of breast cancer- St. Gallen classification

Subtypes of breast cancer	Er and Pr	Her-2	Ki67
Luminal a	Er + and/or pr +	Her-2-	Ki67<14%
Luminal b with her-2 negative	Er+ and/or pr+	Her-2-	Ki-67≥14%
Luminal b with her-2 positive	Er + and / or pr +	Her-2 +	Any ki-67
Her-2 enriched	Er-, pr-	Her-2 +	Any ki-67
Basal-like (triple negative)	Er-, pr-	Her-2-	Ck5/6 + and/or egfr +

Subtypes are characterised based on tumour size, lymph nodes involvement, histologic subtype, the persistence of receptors, lymphovascular invasion, the presence of p53 and stage, and are presented in Tables 2-9.

Table 2: Characteristics of subtypes according to the age of the patients

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	
Number	77	91	70	25	27	290	
(%)	(26.55%)	(31.38%)	(24.14%)	(8.62%)	(9.31%)	(100%)	
Mean age (y)	57.83	58.83	57.64	55.27	56.74	57.56	X=57.6 (y)

From our analysis, we found that:

- Luminal A was present in 77 (26.55%),
- Luminal B HER2 negative was present in 91 (31.38%),
- Luminal B HER2 positive was present in 70 (24.14%),

Table 3: Characteristics of subtypes according to the size of the tumour

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Tumour size							
Tis	6	2	3	2	0	13 (4.48%)	
T1a	11	11	12	1	2	37 (12.76%)	
T1b	8	3	3	1	1	16 (5.51%)	
T1c	9	17	12	7	3	48 (16.55%)	
T2	37	43	36	9	18	143 (49.31%)	
T3	3	6	2	2	2	15 (5.17%)	
T4	3	9	2	3	1	18 (6.19%)	1.0
Number	77	91	70	25	27	290	

- HER2 enriched was present in 25 (8.62%) and
- Basal-like (or triple negative) was present in 27 (9.31%) patients.

Table 4: Characteristics of subtypes according to the size of a tumour and lymph nodes involvement

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Mean tumor size (mm)	29.3	31.8	27.2	31.3	35.0	X = 30.3	
Axillary LN status							
N0	41	28	26	14	10	119 (41.03%)	
N+	36	63	44	11	17	171 (58.97%)	0.99
Number	77	91	70	25	27	290	

Discussion

Breast carcinoma is a heterogeneous disease with several clinical and histopathological presentations, which present different gene expressions in several subtypes and molecular profiles, hence giving different predictive and prognostic characteristics for the patients. Gene expressions were analysed using DNA microarrays. Due to the cost of DNA analysis, the use of immunohistochemical analysis of markers, which have been used as surrogate tools for defining subtypes of breast cancer, was generally accepted. According to the 2011 St. Gallen consensus conference, 5 subtypes of breast cancer were defined using the

presence of receptors on the surface of the tumour cell, and the measuring values of Ki67 [7] [10] [11].

Table 5: Characteristics of subtypes according to histologic subtype

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Histologic subtype							
Ductal	59	76	58	20	24	237(81.72%)	
Lobular	11	6	7	2	1	27 (9.31%)	
Other	7	9	5	3	2	26 (8.97%)	0.99
Number	77	91	70	25	27	290	

Many of the prognostic factors predicting the disease are very well-known, and so is their biological mode of action and how they work to spread the disease in the body. Estrogen receptors are on the surface of the tumour cell, so once estrogen binds with the estrogen receptors, it activates many processes in the cell and stimulates growth and cell division. Hence, estrogen stimulates tumour growth. Giving drugs that block estrogen receptors or drugs that block estrogen synthesis can stop the tumour growth.

Table 6: Characteristics of subtypes according to histological grade

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Histologic grade							
1	6	3	4	0	3	16 (5.52%)	
2	58	56	49	19	20	202 (69.65%)	
3	13	32	17	6	4	72 (24.82%)	0.99
Number	77	91	70	25	27	290	

The same situation applies to the presence of HER-2 neu receptors. HER-2 is a membrane tyrosine kinase and oncogene that is overexpressed and gene amplified in about 20% of the breast cancer cases. When activated it provides the cell with potent proliferative and anti-apoptosis signals, and it is the major driver of tumour development and progression of breast cancer.

Table 7: Characteristics of subtypes according to the persistence of receptors (estrogen, progesterone and HER2 neu)

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Estrogen receptors							
Positive	72	86	57	0	0	215 (74.14%)	
Negative	5	5	13	25	27	75 (25.86%)	1.0
Number	77	91	70	25	27	290	
Progesterone receptors							
Positive	73	88	65	0	0	226 (77.93%)	
Negative	4	3	5	25	27	64 (22.07%)	1.0
Number	77	91	70	25	27	290	
Her 2 neu receptors							
Positive	0	0	70	25	0	95 (32.76%)	
Negative	77	91	0	0	27	195 (67.24%)	1.0
Number	77	91	70	25	27	290	

Overexpression will activate many pathways in the cell, resulting in uncontrolled cell growth and division, causing the tumour to grow uncontrollably. Target drug delivery, monoclonal antibody trastuzumab (Herceptin), will block these receptors, and control a tumour. Moreover, giving chemotherapeutics that interact with the rapidly

dividing cells will control a tumour. Ki67 is the factor that shows the proliferative activity of tumour cells. Ki67 correlates with the S phase of the cell cycle and with the mitotic activity. A normal breast cell has a proliferative activity of 3% (3% of the cells are in the dividing stage).

Table 8: Characteristics of subtypes according to the persistence of p53, LVI (lymphovascular invasion) and values of Ki67

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
P53							
Positive	27	49	29	11	15	131(45.18%)	0.99
Negative	50	42	41	14	12	159 (54.82%)	
Number	77	91	70	25	27	290	
LVI							
Positive	22	45	27	11	12	117 (40.34%)	0.99
Negative	55	46	43	14	15	173 (59.65%)	
Number	77	91	70	25	27	290	
Ki67							
Up to 14%	77	0	36	4	9	126 (43.44%)	1.0
More than 14%	0	91	34	21	18	164 (56.56%)	
Number	77	91	70	25	27	290	

Higher Ki67 index correlates with young age, larger tumours, positive lymph nodes, negative estrogen receptors and positive HER-2 receptors [12]. An activity that is higher than 14%, at some studies large than 20%, shows aggressive tumours with poor prognosis and shorter overall survival [10] [11] [12].

Table 9: Characteristics of subtypes according to the stage of the disease

	LA	LB-Her2-	LB-Her2+	HER2+	TN	Total	p
Stage							
0	1	2	0	0	0	3 (1.03%)	1.
IA	16	6	11	7	3	43 (14.83%)	
IB	3	4	2	0	0	9 (3.10%)	
IIA	26	26	18	6	7	83 (28.62%)	
IIB	13	15	18	6	8	60 (20.69%)	
IIIA	7	17	13	2	4	43 (14.83%)	
IIIB	4	5	2	2	2	15 (5.17%)	
IIIC	7	16	6	2	3	34 (11.72%)	
Number	77	91	70	25	27	290	

Knowing the subtype:

- we can predict the biology of a tumour and its future behaviour;
- we can predict the prognosis of the disease;
- we can plan a targeted therapy for some of the subtypes.

Knowing the prevalence of subtypes in one population can help plan a general therapeutic approach [16].

Some authors define 4 subtypes: Luminal A, Luminal B, HER-2 enriched and basal cell (triple negative) (Valejos, Carey), other authors define 6 subtypes: Luminal A, Luminal B, HER2+, basal-like (triple negative), normal breast cell-like and Claudin-low (Eroles), but the most frequently used classification encompasses 5 subtypes [7] [13] [14] [17]. In practice, breast subtypes are defined by detecting the presence of estrogen, progesterone and

HER-2 neu receptors on the surface of the malignant cell using immunohistochemical assays. Knowing that the presence or absence of receptors on the surface of breast cancer cell is conditioned by gene mutations and overexpression, subtypes can also be detected by assessing the gene expression. This is why the term genotype of breast cancer is cited in the literature.

The most frequent type is Luminal A which is found in 50-72% of the patients with breast cancer. Patients with this type of cancer have the best prognosis, i.e. low proliferative index, good differentiation, with the lowest risk of local recurrence and relapse [13] [15] [16] [17] [18].

However, there are different values registered in literature regarding this subtype: Italy 34%, Saudi Arabia 3.9%, China 65.3% and Japan 71% [24] [25] [26] [27].

The suggested therapy for these patients is third-generation aromatase inhibitors in postmenopausal women, selective estrogen receptor modulators (like tamoxifen) and selective estrogen receptor modulators (like fulvestone) [13] [15] [16] [17] [18].

In our examination, Luminal A type was detected only in 26.55% of the patients.

Luminal B subtype is characterised with positive estrogen receptors, with positive or negative HER2 receptors, with a higher Ki-67 value of over 14%, which in both types of Luminal B gives worse prognosis than Luminal A subtype.

Luminal B type is found in 10-20% of the patients with breast cancer, and in our examination, the HER-2 negative was detected in 31.38% and HER-2 positive in 24.14 % of the patients. Literature references are as follows: Italy 36%, Egypt 24.6% [24] [28]. This shows that most of the tumours in our group are aggressive. Luminal B subtype is much more aggressive than the Luminal A subtype and is characterised by poor differentiation, more frequent bone metastases and with a worse prognosis. Many authors suggest that patients in this subtype are younger patients with bigger tumours, with positive nodal status and higher N stage [16]. Given the presence of larger tumours and the advanced stage of the disease, Luminal B findings are more frequent in our study. Regarding patient's age, there was no difference found between Luminal A and Luminal B types; however, in our study, Luminal B prevailed in older patients [13] [15] [16] [17] [18].

The suggested treatment is with tamoxifen, but also chemotherapy in neoadjuvant and adjuvant courses [13] [15] [16] [17] [18].

HER-2 enriched subtype is found in 15-20% of the patients. In our group, it was detected in 8.62% of the patients. This subtype is characterized by high proliferative index and poor differentiation in most of

the patients, and p53 mutations are very often detected. This is a very aggressive type of a tumour, with only 12% of the patients surviving 10 years [13] [14] [16] [17] [18].

The suggested treatment requires target HER-2 therapy with monoclonal antigen trastuzumab (Herceptin) which changes the prognosis. This therapy needs to be combined with chemotherapy in the neoadjuvant and adjuvant protocol. Treatment with trastuzumab in combination with DM1 is also possible [13] [15] [16] [17] [18].

Subtype Basal-like (triple negative) is characterised with larger tumours, poor differentiation, high mitotic index and tumour necrosis. This type is found in 10-20% of the patients. A similar subgroup of this subtype is Claudin-low, where the only differentiation is the difference in EGFR and the fact that this type is found in 12-14% of the patients. Both subgroups have bad prognosis, poor differentiation and high mitotic index. Very often metastases in visceral organs, lungs and CNS are detected. This type has the worst prognosis, and very often the disease relapses in the first three years, and p53 mutations are very often detected [13] [14] [15] [16] [17] [18] [34].

In our study, the triple negative was detected in 9.31% of the cases.

The suggested therapy is chemotherapy, but also PARP inhibitors (poly ADP ribosome polymerase inhibitors) like olaparib in detected BRCA1 or BRCA2 mutations [14] [15].

The normal breast cell-like subtype that is defined by some authors is found in 5-10% of the patients. This type doesn't respond to neoadjuvant therapy, only to adjuvant chemotherapy protocol. Often, it is well-differentiated with low proliferation index and is characterised by median overall survival [14].

We registered the difference in frequencies among our subgroups and those cited in the literature. This explains the heterogeneity of breast cancer across the world. It is cited in the literature that some subtypes are more frequent in certain races (triple negative is more frequent in African-American women) [13] [19]. Knowing the prevalence of subtypes in one population, we can plan a general therapeutic approach [16].

There is no significant difference in age regarding subgroups of breast cancer, patients range between 52,72 and 58,83 years, except in Luminal B HER negative and HER enriched subgroups ($P = 0.0255$). Patient's age in subgroups of breast cancer is shown in Figure 1.

The mean size of tumours in different subgroups ranges between 27.18 and 35 mm. The biggest tumour diameter was found in the triple negative subgroup, but there were no significant

differences in the whole group, and also between subgroups. The same situation applies to the tumour diameter defined as T stage with no difference in the position between subgroups.

High differentiation values (G3-low differentiation) of the malignant cell in Luminal B subtype of breast cancer were detected in 35.16% of the patients. In triple negative subtype, G3 was detected only in 14.8% of the patients, which is contrary to the case results reported in many studies in the literature [13] [14] [17].

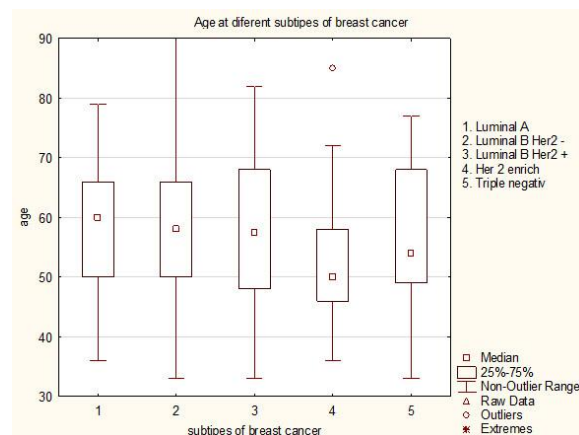


Figure 1: Patients age according to subgroups of breast cancer

Regarding lymphovascular invasion, the highest values were detected in Luminal B HER-2 negative, i.e. in 49.45% of the patients, which suggested invasive and aggressive subtype of breast cancer, with a tendency for lymphatic spread and higher frequency of axillary lymph node involvement (69.23%). The relatively low values of lymphovascular invasion in triple negative subtype are interesting to comment (44.44%), with low axillary lymph node involvement (62.96%), which correlates with the findings in the literature. Knowing the aggression of triple negative subtype of breast cancer, we can conclude that the spreading of malignant cells follows other pathways other than lymphatic [14] [17] [30] [31] [32] [33] [34] [35].

Regarding the values of Ki67, the factor that suggests the proliferative activity of the tumour, its aggression and biology, values higher than 14% were detected in Luminal B HER negative subtype (in all patients), but also in HER-2 enriched and triple-negative subtypes. On the contrary, no values higher than 14% were detected in Luminal A subtype, which suggests that this subtype is less aggressive [13] [14] [15] [16] [17] [18].

The patterns of distribution of cells positive for estrogen, progesterone and HER-2 neu receptors were significantly different in subgroups, which is normal because we know that these receptors are main factors for defining the different subtypes of breast cancer [7] [14].

Knowing the subtype of breast cancer, in addition to the histological type and TNM stage, can suggest further prognosis of the disease, detect the spreading and find where metastases can appear later, but can also suggest further therapeutic approach [15].

Also knowing that all factors that determine breast cancer subtypes can be evaluated from core biopsy materials before starting treatment, some subtypes (the more aggressive ones) can be treated with adequate drugs in neoadjuvant protocol [14].

In conclusion, detecting the subtype of breast cancer is important for disease prognosis, but also for determining and providing an adequate therapy. Hence, the molecular subtype of breast cancer needs to be determined in a routine histopathological assay.

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