THE HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL PROFILE OF MALIGNANT BREAST TUMORS IN NORTH-EAST ROMANIA

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THE HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL PROFILE OF MALIGNANT BREAST TUMORS IN NORTH-EAST ROMANIA (Abstract): Studies using gene expression profiling have identified five major subtypes of breast cancer, apart from the traditional hormone receptor positive or negative ones: luminal A, luminal B, the HER2 group, the basaloid carcinoma group and the "normal breast-like" group. **Material and methods:** In this retrospective study, 281 patients admitted to the Regional Institute of Oncology Iasi and the Clinical Hospital of Obstetrics and Gynecology Cuza Voda Iasi were included, for whom the oncological records, the operative protocols of the operating room, the unique register of the service were studied; the pathological anatomy and observation sheets were considered for a period of 8 years: 2015-2022. For all 281 invasive mammary carcinomas diagnosed on hematoxylin-eosin staining, they were classified into one of the corresponding histopathological types and subtypes according to WHO criteria. **Results:** The histopathological study allowed: 1. invasive ductal carcinoma NOS (not otherwise specified - non-specific type of invasive ductal carcinoma)-193 cases (68.68%),2. invasive lobular carcinoma-42 cases (14.94%). Immuno-labeling analysis Estrogen receptors (ER) were positive (Allred score ≥ 3) in 61% of cases (169 patients), and progesterone receptors (PR) in 64% of cases (180 patients). Ki 67 immunolabeling analysis highlighted: the presence of positivity in all cases studied, overexpression of the p53 protein in 99 cases and HER 2 immunoassays allowed the highlighting of 42 cases. Identifying cases with p53 protein mutations can select a group of patients with a higher risk of recurrence and death, and testing p53 expression in HER2 positive patients identifies the subgroup with more aggressive tumors that will benefit from a more aggressive treatment. **Conclusions:** The most common histological types were *invasive ductal* carcinoma in 90% of patients. TNM stages was as follows: stage I in 9 cases (3.2%), stage II in 88 cases (31.49%), stage III in 138 cases (48.93%), stage IV in 31 cases (11.03%) ER+/PR- phenotype were more frequent over 50years and tumors larger than 2 cm. Patients under 50 years presented twice as often HER2 positive tumors. Ki67 immunostaining seems to be associated with an unfavorable prognosis (2% of cases in the present study). p53 overexpression occurs most frequently in patients under 50 years, with tumors larger than 2 cm. **Keywords**: BREAST CANCER, DUCTAL CARCINOMA, LOBULAR CARCINOMA, ER, PR, KI67, P53, HER2.

Recently, a variety of molecular techniques, especially the expression profile of certain genes, have redefined the classification of breast cancer from the point of view of prognosis, evolution and expected response to treatment (1).

Studies using gene expression profiling have identified five major subtypes of breast cancer, apart from the traditional hormone receptor positive or negative ones: luminal A, luminal B, the HER2 group, the basaloid carcinoma group and the "normal breast-like" group (2, 3) (tab. I)

Three immunohistochemical markers are used in clinical practice for the molecular classification of breast cancer: estrogen receptors (RE), progesterone receptors (RP) and HER2 expression (4). The best differentiated breast tumors are RE+, RP+ and HER2-, while poorly differentiated tumors are RE-, RP- and HER2+. Exceptions are medullary carcinomas (triple negative), basaloid carcinomas (triple negative) and micropapillary carcinoma (triple positive) (5, 6, 7). Luminal A category carcinomas have a better prognosis, while the basaloid subtype is the most aggressive type of cancer.

TABLE I

The immunophenotype of breast carcinoma

MOLECULAR SUBTYPE	IMMUNOHISTOCHEMICAL PROFILE
Luminal A	RE+ and/or RP+, HER2- and ki67<14% (negative)
Luminal B	RE+ and/or RP+, HER2+ (Luminal group B HER2+)
	RE+ and/or RP+, HER2- high expression ki67 (>14%)
HER 2	RE-, RE+, HER+
Basaloid carcinoma	RE-, RP-, HER2-, CK5/6+ and EGFR+

MATERIAL AND METHODS

In this retrospective study, 281 patients admitted to the Regional Institute of Oncology Iasi and the Clinical Hospital of Obstetrics and Gynecology Cuza Voda Iasi were included, for whom the oncological records, the operative protocols of the operating room, the unique register of the service were studied; the pathological anatomy and observation sheets were considered for a period of 8 years: 2015-2022. The breast tumors was monitored according to age, place of residence, tumor size, tumor location, histopathological type, tumor grading, complications of the disease and material investigated in this study was represented by human material, respectively by breast tissue from patients. For all 281 invasive mammary carcinomas diagnosed on hematoxylin-eosin staining, they were classified into one of the corresponding histopathological types and subtypes

according to WHO criteria.

The aim of immunohistochemical study was to highlight some correlations between the cell markers Ki67, p53, HER2, hormone receptors and the histological grade, the prognosis in breast carcinomas. These pieces of surgical exeresis were fixed in formalin and initially processed by the usual paraffin embedding technique, being brought up to the paraffin block stage. The method used in the *immunohistochemical study* was one of the methods based on soluble immunoenzymatically complexes, called LSAB/HRP (*labelled streptavidin biotin*).

RESULTS

Clinical-statistical study. *The urban/rural ratio* was 1.48, urban residence being found in 167 patients. *Age* The maximum incidence of breast cancer was in the 51-60 age group and included 108 patients, representing 38.43%. The *location* of cancer in the left breast was present in 151 cases(53.73%), in the right breast it was present in 127 cases (45.19%), and bilateral breast cancer was present in 3 cases. *Signs and symptoms* The most common signs and symptoms were breast tumor in 260 patients (92.52%), homolateral axillary adenopathy in 185 patients (65.83%), carcinomatous mastitis in 19 patients (6.67%), arm edema in 11 patients (3.91%), nipple discharge in 5 patients (1.77%), bone metastases in 9 patients (3.2%), liver metastases in 8 patients (2.84 %). *The breakdown by TNM stages* was as follows: stage I was established in 9 cases (3.2%), stage II in 88 cases (31.49%), stage III in 138 cases (48.93%), stage IV was diagnosed in 31 cases (11.03%), the stage was unknown in 5.93% of patients.

Histopathological study. The histopathological study allowed, in a first stage, the classification of the 281 invasive breast carcinomas, according to the WHO classification, into one of the following types and subtypes **: 1.** invasive ductal carcinoma NOS (not otherwise specified - non-specific type of invasive ductal carcinoma) - 193 cases (68.68%)**, 2.** invasive lobular carcinoma - 42 cases (14.94%), **3.** mixed ductal-lobular carcinoma - 14 cases (5.16%), 4. Other types- 26 cases.

Immunohistochemical study. All these tumors were processed immunohistochemically and were smaller or equal to 2 cm in 98 cases and larger than 2 cm in 183 cases.

Immunolabeling analysis for hormone receptors (ER and PR). Estrogen receptors (ER) were positive (Allred score $\geq$ 3) in 61% of cases (169 patients), and progesterone receptors (PR) in 64% of cases (180 patients. Compared to the **histological type,** invasive ductal carcinomas expressed estrogen receptors in 114 cases (58.88%) and progesterone receptors in 122 cases (63.3%), while invasive lobular carcinomas expressed estrogen receptors in 34 cases (80%) and progesterone receptors in 29 cases (70%). Depending on the immunoexpression of hormone receptors, breast carcinomas are classified into four subtypes or phenotypes: **1**. 57% of cases presented both types of receptors with an ER positive/PR positive phenotype.**2**. 32% of cases were completely devoid of hormone receptors having an ER negative/PR negative phenotype. **3-4.** 11% had a heterogeneous phenotype: 7% of cases were ER negative/PR positive, 4% of cases were ER positive/PR negative.

Ki 67 immunolabeling analysis highlighted: the specific marking of Ki 67 is

nuclear and was found in 281 cases. Patients under the *age* of 50 more frequently had a high Ki67 index (over 15% of tumor cells being Ki 67 positive) compared to patients over 50 (60% of cases vs 55.55%); patients with *tumors larger* than 2 cm more frequently had a high Ki 67 index compared to those with *tumors below* 2 cm (71.42% vs 51.43%). Regarding the histological type of the analyzed carcinomas, it was found: the *lobular type* had a low Ki 67 index in all cases; the *ductal invasive type* had a low Ki 67 index in 43.87% of cases and a high index in 56.12% of cases. Related to the histological grade: high-grade tumors (G3) always had a high Ki67 index; low-grade (G1/G2) that had an increased proliferation index in only 8.62% cases.

The analysis of p53 immunolabeling identified the presence of overexpression of the p53 protein (more than 10% of tumor cells labeled with this antibody) in 99 cases (42% of the studied cases, 193 ductal carcinomas and 42 lobular carcinomas). Tumors with p53 overexpression were more frequently encountered in patients under 50 years of age (54 cases, respectively 54.55%) compared to those over 50 (45 cases, respectively 45.45%). p53 positive tumors were more frequently tumors larger than 2 cm. So, 66 tumors among the p53-positive tumors (representing 66.64% of the p53-overexpressing group) were larger than 2 cm in size, while only 33 of the p53-positive tumors (representing 33.33% of the p53-overexpressing group) were larger than 2 cm in size. smaller than 2 cm. Invasive ductal type carcinomas were p53 positive in 85 cases, representing 44.44% of all invasive ductal type cases, and invasive lobular type carcinomas in only 13 cases, representing (30%) of all type breast carcinoma cases invasive lobular Regarding the histological grade, most cases with p53 protein overexpression (73 cases, respectively 73.3%) were of high grade (G3) and only 51 cases (28.33%) had low histological grade (G1 and G2). The immunohistochemical overexpression of p53 was thus found in 46.66% of the 35 HER2 positive cases (score 2+ and 3+) and only in 96 cases (40.85%) of the 200 HER2 negative cases (score 0 and 1+). p53 overexpression (corresponding to an increased amount of p53 protein determined by IHC) was more frequent in HER2-positive breast carcinomas compared to HER2-negative ones (46.66% vs 41.17%).

The analysis of the HER 2 immunoassay allowed the highlighting of 42 cases (representing 14.94% of the studied cases) of invasive breast carcinoma positive for HER 2. Of these, 28 cases (representing 66.66%) were weakly positive (score 2+), and 14 cases (representing 33.34%) were interpreted with a score of 3+ (positive) according to the criteria (Group Biology HER2/CEP 17) Correlating the HER2 immunolabeling of tumors with their histological type, it was observed that 4 cases of lobular carcinoma (10% of lobular carcinomas were HER2 positive) were moderately HER2 positive (score 2+). All other 38 HER 2 positive cases were of ductal type (24 cases with score 2+ and 14 cases with score 3+). Thus, 15.55% of invasive ductal carcinomas were HER2 positive. Following the correlation of HER2 immunomarking with morpho-clinical parameters, it was observed that HER2 positive tumors belonged slightly more frequently to patients under 50 years of age (22 cases, respectively 52.38%) compared to those

over 50 (20 cases, respectively 47, 62%). HER2 positive tumors were more frequently tumors larger than 2 cm. Thus, 22 tumors larger than 2 cm (representing 52.38%) were HER2 positive, while 20 tumors smaller than 2 cm (representing 47.62%) were HER2 negative. Regarding the histological grade, the majority of HER2 positive cases (34 cases, respectively 80.9%) were of high grade (G3) and only 8 cases (representing 19%) had low histological grade (G1 and G2).

DISCUSSION

The clinical study was conducted over a period of 8 years, between 2015-2022 and included 281 patients with breast cancer.

Age. The maximum incidence of breast cancer was in the 50-59 age group and included 79 patients (28.11%). The older the age, the greater the risk of breast cancer. The reason for this reality seems to be related to the existence of multiple somatic mutations (multiple event theory) for breast cancer genes as the population ages (12) (tab. II).

TABLE II

Breast cancer incidence according to age (13, 14, 15, 16)

Author	Patients group	Age (years)	Incidence of breast cancer
Andrew McGuire	26 countries	50-59	71-73%
Allen S. Lichter	247	50-60	51.0%
Edward Obedian	1,029	> 50	67.9%
Coral A. Quiet	826	> 55	50.0%
Richard McQuellon	115	> 57	>50.%

Histological grading The distribution of tumors according to histological grading was as follows: histological grading G1 in 22% of cases; histological grading G2 in 50% of cases; histological grading G3 in 23% of cases. Histological grading is usually based on the criteria established by Bloom. These include tubule formation, mitoses, and nuclear characteristics (size, shape, and hyperchromatism).Black *et al Schuh* (17) divided the nuclear grades into three groups and counted them in reverse order of the histological grading: • nuclear grade1- anaplasia, • nuclear grade2- intermediate differentiation • nuclear grade3- well differentiated.

TNM staging was possible in 94.66% of cases, and in 5.93% of patients the stage was unknown. The breakdown by TNM stage was as follows: • stage I was established in 9 cases (3.2%),• stage II in 89 cases (31.49%),• stage III in 137 cases (48.93%),• stage IV was diagnosed in 31 cases (11.03%),• the stage was unknown in 5.93% of patients.

Histological study. The classification of these invasive carcinomas into the various types according to the WHO criteria demonstrated that an overwhelming number of cases (193 cases, respectively 68.51%) were represented by NOS-type invasive ductal carcinoma, these cases being followed by invasive lobular carcinoma, which was diagnosed in 42 cases, representing 14.94% of all infiltrative breast carcinoma cases studied. *Invasive*

ductal breast carcinoma NOS. It is the most common invasive breast carcinoma, representing between 40% and 75% of invasive breast carcinomas. By definition, the diagnosis of ductal carcinoma NOS is a diagnosis of exclusion, this carcinoma being a malignant epithelial tumor that cannot be classified into any of the special types of invasive breast carcinoma (20). According to the Nottingham score, NOS ductal carcinomas were classified into: NOS G1 carcinomas (48 cases - 17.08%), NOS G2 carcinomas (132 cases - 46.97%) and NOS G3 carcinomas (101 cases - 35.94 %). The separation of breast carcinomas into the 3 histological grades is arbitrary and artificial, because these tumors actually present a continuous scale of malignancy (20).

Lobular carcinoma. It was first introduced by Foote and Stewart in 1941. Invasive lobular carcinoma is the most common type of invasive breast carcinoma after invasive ductal carcinoma, accounting for 5-15% of all breast carcinomas in Europe and the United States, while in Japan this type of carcinoma represents only 1-4% of all breast cancers (21).

The classic subtype of infiltrative lobular carcinoma accounts for approximately 40% of all infiltrative lobular carcinomas (*22*). According to the Nottingham score, invasive lobular carcinomas were classified into carcinomas: G1 16 cases - 19.04%, G2 51 cases - 60.71%, G3 17 cases - 20.23%. Similar results were obtained by specialists and published in recent studies. Thus, using the criteria of the Nottingham score, classified invasive lobular carcinomas in grade 1 - 20% of cases, grade 2 - 66% of cases and grade 3 - 14% of cases and showed that the histological grade of invasive lobular carcinomas (24).

Immunohistochemical study. Hormone receptors. In this study, 61% of invasive breast carcinomas showed estrogen receptors, and progesterone receptors were detected in 64% of cases, this being in accordance with recent data from the literature that records the presence of ER in 63% of patients with invasive breast carcinoma and a PR in 65% of them (25). The majority of breast carcinomas expressed both types of hormone receptors with an ER+/PR+ phenotype (57% of cases) and were followed in frequency by tumors with absent hormone receptors and an ER-/PR- phenotype (32% of cases). Specialist studies cite that approximately 50% of invasive breast carcinomas express both types of hormone receptors, and approximately 25% lack estrogen and progesterone receptors (26). In the present study, the ER+/PR- phenotype was detected in 4% of tumors. Thus, the average level of estrogen receptors in ER+/PR- tumors is only about half that of ER+/PR+ tumors (27). The Ki-67 antigen is a non-histone protein useful for identifying proliferative cells that, not having phase specificity, is expressed in all active phases of the cell cycle (Ki-67 is not expressed in the G0 phase) (28).

Although there are currently several antibodies that can be used on paraffin sections (MM1, NCL-ki-67p, Rah Ki-67), studies show that, nevertheless, the MIB-1 antibody has the highest sensitivity, offering the best visual staining, the reproducibility of the staining index being equally good for any of these antibodies (29). Its distribution by immunohistochemical methods is limited to its appearance in the late G1 phase. Therefore, Ki-67 is recognized as an indicator of cellular mitotic

activity. An increase in Ki-67 expression indicates an increase in cellular mitotic activity and proliferation*(*30). Immunohistochemical expression of Ki 67 correlates well with growth fraction in various models and does not appear to be expressed during the DNA repair process .(31) In breast cancers, Ki67 is used to categorize patients into categories with favorable and unfavorable prognosis correlating with the clinical response to chemotherapy (32). Relative to the histological grade, high-grade tumors (G3) always had a high Ki 67 index, compared to low-grade tumors (G1/G2) which had an increased proliferation index in only 8.62% of cases. All studies that reported correlations of the KI 67 index with the histological grade of the tumors showed that tumors with a high histological grade have an increased proliferative activity compared to those with a low grade (33, 34).

The oncoprotein p53 is a phosphoprotein that has the role of "guardian of the genome", i.e., monitoring the integrity of DNA during cell division. The protein product of normal (wild-type) alleles of the p53 gene negatively regulates cell growth and proliferation, blocking cells in the G1 phase of the cell cycle. In the present study, the overexpression of the p53 protein was found in 42% of the breast carcinoma cases studied, a result well correlated with the data published in the specialized literature that vary between 16% and 48% of p53 positive cases in invasive breast carcinomas (36). Analyzing the overexpression of the p53 protein in the cases included in this study, we found that it was more frequent in patients under 50 compared to those over 50 (54.76% *vs.* 45.24%), with tumors larger than 2 cm (66.64% *vs.* 33.33). Regarding the type and histological grade of the analyzed invasive breast carcinomas, p53 overexpression was more frequently associated with the invasive ductal type compared to the lobular one (95.24% *vs.* 4.76%) and with poorly differentiated tumors (G3) compared to the well/moderately differentiated ones (28.57% *vs.* 71.43%). These results are consistent with recent studies that demonstrated that breast tumors with a high amount of p53 (measured by IHC) are significantly correlated with high histological and nuclear grade (*36*) and they are much less often lobular (37). p53 immunopositivity correlated with the absence of expression of estrogen and progesterone receptors (61.9% of cases were devoid of estrogen receptor expression *vs.* 38.1% of cases showing estrogen receptor immunoexpression) also 54.76% of cases with p53 overexpression had been negative for PR *vs.* 45.24% of positive PR cases.

Co-expression of p53 and HER 2 was found in 7% of invasive breast carcinoma cases included in this study. The rate of breast cancers that coexpress p53 and HER 2 was reported, in previous studies, as varying between 7% and 19.5% of all breast cancers examined (38).

HER2 protein. The product encoded by the HER2 gene is a transmembrane protein of approximately 185 kD that functions as a tyrosine kinase receptor, called the HER2 protein, p185 or c-erbB-2. HER2 is involved in cell differentiation, proliferation, adhesion and motility (39), occupying a key position at the level of the mitogenic signal transduction cascade both in the case of normal and tumor cells. Thus, in addition to hormone receptors, HER2 represents the most accepted prognostic and predictive factor of response or resistance

to treatment and the potential of using new treatments (Herceptin) (40).

In this study, 15% of the analyzed group's tumors were positive for HER2, of which only 5% of the cases overexpressed the HER2 protein with maximum intensity (score 3+). The overexpression rate with maximum intensity of the HER 2 protein was lower in the studied group probably due to the fact that the poorly differentiated tumors (grade G3) were much less (40%) compared to the well and moderately differentiated ones (60%). This fact supports the hypothesis that less aggressive breast tumors have a lower rate of HER2 overexpression and suggests the association between HER2 expression in the tumor and the degree of its malignancy. HER2 positive tumors were more frequently tumors larger than 2 cm. Thus, 8 tumors larger than 2 cm (representing 53.33%) were HER2 positive, while 7 tumors smaller than 2 cm (representing 46.67%) were HER2 negative. Various studies did not find an association between HER 2 overexpression and the sizes of breast tumors analyzed (41,42). Correlating the HER2 immunolabeling of tumors with their histological type found that 10% of lobular carcinomas were HER2 positive. All other HER 2 positive cases were of ductal type (with score 2+ and 3+). Thus, 15.55% of invasive ductal breast carcinomas studied were HER2 positives. Recent data suggest that HER2 overexpression is significantly more frequent in ductal than in lobular carcinomas (43).

Co-expression of HER 2 with that of ER was found in only 10.52% of breast tumors with ER+/PR+ phenotype and in 25% of breast tumors with ER+/PR- phenotype). It should be mentioned here that the presence of hormone receptors in HER positive patients does not affect the benefits of trastuzumab therapy (44).

CONCLUSIONS

The maximum incidence of breast cancer was in the 50-59 age group and included 158 patients (28.11%). The most common histological types were *invasive ductal* carcinoma in 90% of patients, *invasive lobular* carcinoma in 4.6%, carcinoma in situ in 0.8% .

The distribution by TNM stages was as follows: stage I in 9 cases (3.2%), stage II in 88 cases (31.49%), stage III in 138 cases (48.93%), stage IV in 31 cases (11.03%), the stage was unknown in 5.93% of patients. *Invasive lobular carcinomas represented, after invasive ductal carcinoma* type NOS, the most frequent (14.94% of cases) type of breast carcinoma and were presented under a very wide range of variants (classic, solid, alveolar, tubulolobular, pleiomorphic, histiocytoid and with signet ring cells). *The histological grading*. A large number of patients (101 patients representing 35.76%) were identified who presented a high histological grade (G3) correlated with a poor prognosis, compared to patients whose tumors were well differentiated (G1, 48 cases, representing 17.08%).

The subgroup of invasive breast carcinomas is that with the ER+/PR- phenotype, more frequent in patients over 50 who had tumors larger than 2 cm. The group of patients under the age of 50 presented twice as often HER2 positive tumors. Overexpression with maximum intensity of the HER2 protein is more frequently associated with poorly differentiated tumors and those without hormonal receptors. Ki67 immunostaining seems to be associated with

an unfavorable prognosis (2% of cases in the present study). Cell proliferative activity and hormone receptor status were frequent in an inverse relationship. p53 overexpression occurs most frequently in patients under 50 years of age, with tumors larger than 2 cm, of invasive ductal type and with high histological grade. The co-expression of the p53 protein with HER2 has always been associated with an extremely high proliferative activity of the respective tumors. Cases with p53 protein mutations can select a group of patients with a higher risk of recurrence and death.

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