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# Tumor-infiltrating lymphocytes and levels of PD-L1 and BRCA protein expression may identify patients with breast cancer with a higher rate of *BRCA1* mutations

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## Introduction

Breast cancer (BC) is a heterogeneous disease, treated as per the predictive role of immunohistochemistry (IHC) identifiers as estrogen/progesterone and HER2 receptor proteins. Deeper molecular classification (MC) identifies molecular subtypes according to the gene-expression profiles with different molecular genetic alterations and biological features, present in the different subtype. Overlap between IHC and MC exists, though incomplete. We aimed to identify overlap between IHC and MC and identify patients with basal-like subtype of BC. We hypothesized that the rates of the tumor expression of breast cancer-related protein 1 (BRCA1), the type of tumor-infiltrating lymphocytes and the expression of programmed death ligand 1 (PD-L1) by immune cells vary among different subtypes of BC.

## Material and methods

Parafin-embedded samples from 100 patients with primary invasive BC were analyzed and expression levels of estrogen and progesterone receptors, HER2 status and Ki-67 were assessed via IHC, defining four groups – luminal A-like, luminal B-like, HER2-positive non-luminal and triple negative (TN). The primary endpoint of our study was to identify via IHC with CK5/6 and 17 basal-like subtype of BC amongst others and to describe specific clinicopathological features together with protein expression of BRCA1 and PD-L1 and tumor-infiltrating lymphocytes, using CD20, CD3, CD4, CD8 and FoxP3.

## Results

Basal-like BC were predominantly characterized as triple negative by IHC ($p < 0.05$) and were more frequently seen among special BC subtypes as compared to no special type (NST), with $p = 0.036$. Their immune response was represented mostly by high concentration of intratumoral cytotoxic CD8(+) T-lymphocytes ($p < 0.05$) and stromal PD-L1-positive immune cells ($p = 0.008$). In these tumors, absence of expression of BRCA1 protein was more frequent ($p < 0.001$). Basal-like subtype of BC with absent expression of BRCA1 is associated with worse <5-year survival ($p = 0.001$ and $p = 0.017$, respectively).

**Conclusion**

The use of IHC can establish a basal-like BC, its immune response and expression for a BRCA-related protein, as the lack of the latter possibly reflects dysfunction in the corresponding gene.

**Key words:** breast cancer, PD-L1, BRCA1, tumor-infiltrating lymphocytes

## Introduction

Currently, there are conflicting data about the effects of the interaction of tumor-infiltrating lymphocytes (TILs) and tumor cells, the importance of immune ''checkpoint'' pathways in the regulation of the immune response (IR) as well as their role in patients with breast cancer (BC), having impaired function in *BRCA1* and *BRCA2* genes [1, 2].

The complexity of the problem is due to the heterogeneity of the primary tumor in this type of neoplasm [3–5]. Different groups of BC are characterized by different molecular and genetic alterations. The defined molecular types – luminal A and B, basal and HER2-positive, are subtypes with different prognosis and response to therapy. The basal subtypes, expressing basal cell cytokeratins such as CK5/6 and CK17, are often characterized by immunohistochemistry (IHC) as triple negative (TN) phenotype. Basal-like (BL) subtype is characterized by the most unfavorable prognosis and genetic instability due to a multitude of mutations, including *BRCA1* and *BRCA2* genes [6]. On the other hand, mutational products are perceived by the body as neo-antigens, inducing IR and transforming these types of tumors into more immunogenic neoplasms, characterized by a more pronounced inflammatory infiltrate in the stroma, tumor and non-neoplastic tissue. However, whether

the detected in the tumor immune cells (IC) are active with an effective antitumor IR or whether they are suppressed as a result of interaction with the tumor cells (TC), or due to the involvement of immune checkpoint inhibitory pathways remains questionable. Further clarification of this may increase the possibility of desired immune modulation [1, 7].

Impaired function of the *BRCA1* and *BRCA2* genes due to germline/somatic mutations and/or epigenetic mechanisms is involved in the pathogenesis of some hereditary and sporadic cases of BC. Using IHC it is possible to establish correlations in the expression of relevant proteins, reflecting the altered activity of their genes [8, 9].

The aim of our study was to determine basal-like subtype of BC, its tumor expression of BRCA1 protein, the predominant type of lymphocytes and the expression of the programmed cell death- ligand (PD-L1) by IC, using IHC method.

## Materials and methods

### *Patients*

This project have been approved by the Ethics commission at the Medical University, Pleven. After anonimization and coding of patient data, no personal information of the studied patients can be identified.

We retrospectively analyzed 25 IHC characterised as luminal A-like, luminal B-like, HER2-positive and TN primary breast cancer samples – a total of 100 BC samples. A random selection from a list of archival tumor blocks at the University Hospital Georgi Stranski and the department of pathology was done. All paraffin-embedded tumor blocks were rechecked in order to confirm the availability of sufficient quantity of tumor tissue. Tumor blocks, that had enough remaining tissue with no risk of tumor depletion after the planned research were selected for the analysis.

The list of patients consisted of two hundred and ninety samples with a diagnosis of primary BC for the period 01.01.2011–05.01.2015. Clinical description of inflammatory BC or other inflammatory or inflammation reactions or conditions within the breast were not considered eligible. Core biopsies or tumor samples after systemic therapy were also considered ineligible for the purposes of our analysis. The selected patients of each subtype of breast carcinoma are few, because a small number of cases diagnosed during the indicated period met the study's inclusion and exclusion criteria. We followed the overall 5-year

survival of all of them, but we did not have access to information on their progression-free survival.

Standard stained by hematoxylin/eosin (HE) slides from the archival tissue were examined with additional IHC tests, consisting of staining for estrogen receptor (ER), progesterone receptor (PR), HER2 and proliferation index Ki-67. One slide per each tumor was selected to assess the expression of CK5/6, CK17, BRCA1, PD-L1 and TILs subtypes (B-lymphocytes – CD20(+), T-lymphocytes – CD3(+), T-helpers – CD4(+), T-cytotoxic cells – CD8(+) and regulatory cells – FoxP3(+)) in staining with IHC. In our cases, the BRCA status determined by genetic analysis is not done and we cannot correlate it definitely with the protein expression.

A formulary, listing the anonymized data, was specifically elaborated for this analysis. We collected and filled in data for demographics (sex and age), clinical characteristics (type of surgical intervention and clinical staging), pathological description (grade of differentiation (G), morphological description, lymph node (LN) involvement, lymphovascular invasion (LVI) and IHC for ER/PR, HER2 and Ki-67) and 5-year survival.

***Histological examination as per the current recommendations for the period of the diagnosis***

Classification of the BC was done as per the 4$^{th}$ edition of the WHO histology classification [10]. The Nottingham grading system (Ellston and Elis, 1991) was applied in order to assess the grade (G) of the invasive cancers [11]. Staging of the disease was done as per the 7$^{th}$ edition of the Tumor-Node-Metastasis (TNM) classification by the American Joint Committee on Cancer (AJCC) staging manual and the 2010$^{th}$ Union for International Cancer Control (UICC) [12].

IHC and expression of proteins for ER/PR, HER2 and Ki-67 was used to histologically classify among the four pathological subtypes of BC as per the 2013 St. Gallen's expert recommendations for the management of early BC [13]. IHC assessment of ER/PR and HER2 was done as per the ASCO/CAP recommendations [14, 15]. The IHC levels of Ki-67 expression were interpreted as per the Working Group on BC recommendations [16].

***Immunohistochemistry***

Silanized microscopic slides 7109-A from sections with a 3–4 μm thickness were done from the identified for the analysis formalin-fixed and paraffin-embedded (FFPE) tumor blocks.

A visualization EnVision™ FLEX, High pH (DAKO) system and AutostainerLink 48 technique (DAKO) were used for the preparation of the IHC slides. All tissue samples were stained using the following primary antibodies:

- CD3 (polyclonal antibody, Rb, dilution 1:50, Dako, DK),
- CD4 (4B12 clone, mo, dilution 1:50, Dako, DK),
- CD8 (C8 / 144B clone, mo, dilution 1:50, Dako, DK),
- CD20 (L26 clone, mo, dilution 1:200, Dako, DK),
- CK17 (E3, clone, mo, RTU, Dako, DK),
- CK5/6 (D5/16 B4 Clone, mo, RTU; Dako, DK),
- FoxP 3 (236A/E7 clone, mo, dilution 1:100, Bioscience, California, USA),
- PD-L1 (Clone 22C3, monoclonal mouse anti-human PD-L1, dilution 1:50, Dako, DK),
- BRCA1 (MS110 clone, mo, dilution 1:100; Abcam, UK).

At the time of our study, there were no generally accepted recommendations for reporting the markers we investigated. The cut-offs for them were determined by a research team based on the average values of the results obtained for all studied patients.

Immunohistochemistry (IHC) staining with CK5/6 and CK17 antibodies was used to identify basal-like subtype of BC. IHC definition of basal-like subtype was identified when the samples of BC had a positive expression of >60% (cytoplasmic for CK5/6; cytoplasmic and/or membrane for CK17) for both cytokeratins or expression >80% of any of them.

Immunohistochemistry staining for PD-L1 (22C3 clone) was also done and the levels of PD-L1 expression were scored as per the percentage of positivity in immune cells (IC). PD-L1 staining was considered positive at magnification ×20 if membrane and/or cytoplasmic staining in lymphocytes directly associated with the response was detected in the invasive tumor. Cut-off, accepted for positivity, was 1%.

BRCA protein expression on tumor cells was also assessed by IHC staining with MS110 clone antibody. Detection of nuclear staining in the tumor cells was compared to that of normal epithelial cells (in which strong nuclear staining is normal and used as an internal control) and intensity was graded as 1(+), 2(+) and 3(+). The percentage of viable cancer cells and the intensity of marking were largely variable. Negative BRCA1 expression was

considered in case of detection of >20% of viable tumor cells and intensity of 1(+) or in the absence of any staining. Positive expression of BRCA1 was considered if nuclear staining was measured as 2(+) and/or 3(+) in >80% of tumor cells.

Subtyping of immune infiltrates was done by IHC staining with CD20, CD3, CD4, CD8 and FoxP3, detecting respectively B-, T- and T subtypes – helper, cytotoxic and regulatory lymphocytes.

Immunohistochemistry expression for different lymphocyte populations was considered positive if the following expression was detected:

- CD3 – membrane and/or cytoplasmic,
- CD4 – membrane,
- CD8 – membrane and cytoplasmic,
- CD20 – membrane,
- FoxP3 – nuclear staining.

The lymphocytes were subject of immune phenotypisation and were divided into intratumoural and stromal. Their levels were separately calculated, semi-quantitatively graded and further analyzed. Depending on the average number of IHC positive cells, the results were recorded as: 0 (no positive cells), low and high number of TILs subsets.

Using antibodies against CD3, CD4, CD8 and CD20 and positive staining identified TILs both in tumor and stroma. Their respective levels were measured and this was done at high magnification of high power field (HPF) ×400 in 5 randomly selected fields. The interpretation of the results was semi-quantitatively and divided into binary groups: TILs were considered as low in cases of detection of less than 25 IHC positive cells and high if ≥25 IHC-positive cells were measured. Lymphocytes in the tumor and the stroma,stained by the FoxP3, were also differentiated into two groups semi-quantitatively and were counted in minimum 10 tumor fields at 400× HPF magnification: detection of less than 15 FoxP3-positive cells was interpreted as low lymphocyte expression and levels ≥15 were considered as high level of regulatory lymphocyte expression.

***Statistical design and analysis***

The results of the testing of the prespecified biomarkers were summarized and data was statistically analyzed using IBM SPSS Statistics 25.0 and MedCalc software Version 14.8.1.

Descriptive statistics was used and categorical features were summarized with frequencies and percentages. P-values were calculated and values <0.05 were considered as significant.

## Results

### *Patient and tumor characteristics*

Median age of all 100 patients was 63.90 ± 12.17 years and most of them were over 50 years (84.0%) at the time of their diagnosis (tab. I). Included in the study were mainly tissue samples from mastectomy (78%). Invasive ductal carcinoma (IDC) of no special type (NST) was the most common histology in 80.0% of the cases and different special morphological types of IDC were detected in 11.0%:

- mucinous: (n = 4),
- neuroendocrine features (n = 1),
- tubular (n = 1),
- with apocrine differentiation, metaplastic (n = 3),
- with medullary features (n = 1),
- adenoid cystic (n = 1).

Lobular type of histology was identified in 9% of the BC samples. Low and intermediate grade (G1–2) tumors was the most common differentiation degree, detected in 52% of the tumor samples, whereas the remaining samples were G3 tumors (48%). 79% of all patients had tumors larger than 3 cm in size, with most (88% each) having LumB and HER2 subtypes.

The highest percentage (36%) of tumors ≤3 cm were from the LumA subtype group. The majority of patients (72%) were diagnosed in stage I–II, the remaining were stage III (27%) and stage IV (1%). The axillary lymph nodes were not involved by metastatic dissemination in 53% of the patients (pN0) and were positive in the remaining 47%. Lymphovascular invasion (LVI+) was observed in 24% LVI, and it was present in 16.9% of the pN0 patients. The 5-year survival rate of the cohort of all 100 patients was 55%.

All patients included in our study were not treated preoperatively. However, we did not have access to the ongoing therapy of most of them, therefore we did not include this type of information in the clinical data studied.

### *Rates of basal-like subtype among groups*

Basal-like subtype of BC (BLBC) was identified by positivity in CK5/6 and/or CK17 as described above and was found in 18% of all 100 cases with BC. Most BLBC were detected in the group of TNBC (48%) – 12 out of 25 patients, followed by 12% in the HER2-positive group (3 out of 25), 8% in the luminal B-like group (2 out of 25) and the smallest percentage – 4% was in luminal A-like type (1 out of 25) and this distribution of BL cancers was statistically significant ($p < 0.05$) (fig. 1).

If analysed by BC subtype, patients were divided into NST (80%), ILC (9%) and special type IDC (11%). Within the special type the relative rate of BL subtype was significantly higher ($p = 0.036$) compared to those of non-BLBC. With other words, patients with IHC for TNBC have a significantly higher percentage of non-BL subtype in the presence of NST histological type, while in spacial type the relative proportion of those with basal subtype is significantly higher ($p = 0.036$).

***Assessment of immune response in basal-like BC – lymphocyte subtypes and PD-L1 expression***

Immune response (IR) in BLBC was more represented and consisted predominantly of significantly higher rates of intratumoral cytotoxic CD8(+) T-lymphocytes ($p < 0.05$) and stromal PD-L1-positive immune cells ($p = 0.008$) (fig. 2).

***Type of BRCA 1 protein expression in BL-BC***

In BL-BC, absent expression of BRCA1 protein from the tumor cells was more frequently noted ($p < 0.001$) (fig. 3).

***Prognostic significance of the results***

Patients with BL subtype BC (18%) and IHC negative expression of BRCA1 protein (26%) had worse 5-year survival ($p = 0.001$ and $p = 0.017$, respectively) (tab. II and III).

**Discussion**

Knowledge about heterogeneity of primary breast cancer (BC) is continuously evolving and discrepancy between clinical behavior and histologically, molecularly and biologically determined subtype is being largely discussed [1, 17]. There are different risk factors for

development of BC, divided into non-genetic (reproductive and lifestyle-related), genetic (mainly inherited mutations) and epigenetic (leading to genetic dysfunction) [18, 19]. Among the genes, involved in the pathogenesis of this neoplasm, scientific data is mostly available for the breast cancer susceptibility genes type 1 and type 2 (*BRCA1* and *BRCA2*), located in 17q21 and 13q12, respectively. Their normal function in non-neoplastic cells is basically related to the repair of damaged DNA, regulation of the cell cycle, the processes of transcription and replication of DNA, providing genetic stability of the cell. The two genes function in coordination at different stages of implementation, although they are not located on homologous chromosomes [18, 20]. Molecular genetic testing is extensively studied during the last years, but its introduction into the real daily clinical practice will take more time due to its high financial burden. Thus, treatment decision still remain based on IHC markers.

The function of *BRCA1* and *BRCA2* genes may be impaired due to germline/somatic mutation or epigenetic silencing mechanisms (decreased gene expression, decreased *BRCA1* mRNA levels and corresponding protein expression, methylation of the *BRCA1* promoter region, amplification of the *BRCA2* gene, etc.). Such abnormalities may cause deficiencies in the *BRCA*-dependent double-stranded DNA homologous recombination repair. Cells with *BRCA1* and *BRCA2* alterations become dependent on alternative repair mechanisms, and unresolved genetic defects lead to genomic instability with an increased risk of cancer initiation. Women, carriers of a *BRCA1* germline mutation, have and increased oncogenic risk for different cancer localizations: up to 85% lifetime risk for BC, up to 60% for epithelial ovarian cancer (eOC). Elevated oncogenic risk exists in *BRCA2* mutations carriers as well with up to 49% of lifetime risk for BC and up to 18% for eOC [1, 8, 9, 20, 21].

There are conflicting data on the subcellular distribution of the protein product through which the *BRCA* genes performs its functions. It accumulates in the nucleus, but the movement of protein from the nucleus into the cytoplasm has also been found [8]. The complete loss of function of the *BRCA1* gene in mammary epithelial cells is considered to be an accelerator of proliferation and tumor progression. Altered gene activity leads to impaired function with abnormal expression and subcellular distribution of their respective proteins.

There are few publications, related to the use of the IHC method to determine the status of BRCA-related proteins. According to some of them, decreased or absent expression is observed only in tumor cells, but in normal – it is strong and monomorphic [8, 9, 19]. In

our study, BRCA1 expression also showed homogeneous strong nuclear and weak cytoplasmic expression in epithelial cells of terminal duct lobular units in normal breast and in some of these cases – loss of expression in tumor cells was observed.

Breast cancer may be most frequently sporadic and rarely hereditary [22]. Only 5–10% of all BC are inherited and are due to germline mutations in highly penetrating sensitive genes, such as *BRCA1* and *BRCA2*, *PALB2*, T*P53*, *CDH1* and *PTEN*, leading to a cumulative risk of development of this and other neoplasms. However, penetrance is incomplete and depends on various factors, such as the type and location of the mutation, the influence of population and exogenous factors. Only <5% of the familial BC have a mutation in the *BRCA1* and *BRCA2* genes, with the frequency and types of mutations varying by geographical location [18].

Most cases are sporadic and are not the result of a hereditary genetic predisposition. Some of them have characteristics (phenotype) of *BRCA1* and *BRCA2* germ-mutated tumors [1] and are associated with somatic mutations and/or epigenetic alterations that inactivate the *BRCA1* and *BRCA2* genes, the so-called "BRCAness" BC. Epigenetic mechanisms important for the regulation of gene expression may also be involved in hereditary cases, but are more common in sporadic cases [8, 9, 18, 20–23].

*BRCA1* mutated and BRCAness tumors are a heterogeneous group with various pathological and clinical data, molecularly associated with increased genomic instability. Predominant morphological features include invasive ductal (no special type – NST) histological type, tumors with high proliferative index and low differentiation, i.e. with high histological degree (high grade/G3). Often manifest with pronounced necrosis and lymphocyte infiltrate (possibly more immunogenic), medullary characteristics, well demarcated from peripheral non-tumor tissue, negative hormonal receptor status for ER and PR, HER2-negative, without *in situ* component [20, 21, 24–27].

Among the major molecular surrogate subtypes of BC, the TN subtype includes 15–20% of all BC cases. This subtype is most common in patients with *BRCA1* and *BRCA2* mutations or "BRCAness" BC, with 70% of germline *BRCA* mutated tumors being TN and 10–20% of TNBC having germline *BRCA1* and *BRCA2* mutations [1, 7, 8, 17, 23]. TNBC has aggressive clinical behavior and unfavorable morphological characteristics [1, 7, 8, 17, 23]. This reflects a worse prognosis and necessitates the development of targeted therapy and

the establishment of appropriate predictive markers, allowing the selection of patients in whom it would have a more favorable effect.

Fifty–sixty percent of TNBC have a basal-like phenotype in which the molecular and IHC profile shows expression of basal cell or myoepithelial markers (e.g., CK5/6, CK14, CK17, p-cadherin, EGFR, etc.). The majority of these tumors are non-special/ductal type [28]. But most special histological types of TNBC are basal subset [29]. Eighty percent of basal-like carcinomas are TN, but TN and basal-like carcinomas are not synonymous. Basal-like BC have the highest mutational load, including often have a *BRCA1* mutation and *vice versa*, most (about 80%) *BRCA1*-related carcinomas are basal-like [7, 8, 17, 23]. The predominant proportion of basal type of BC have aggressive clinical behavior [6, 28].

Existing similarities between *BRCA1* mutated, TNBC and basal-like BC may be critical for clinical behavior, as well as prognostic and predictive value in patients with impaired function in the *BRCA1* and *BRCA2* genes [1, 23].

In our study there are also similar results regarding TN, basal-like BC and these tumors with lost *BRCA1* IHC expression. The basal-like subtype was also found mainly in TNBC, compared to another surrogate molecular subtypes of BC. Furthermore basal-like BC predominates in the group of other special histological variant compared to NST and lobular type of BC. In addition, we noticed that in the tumor cells of basal-like subtype the negative expression for *BRCA1* is more common, compared to the non-basal category of the tumors, where IHC positivity is often preserved. The disadvantage of our study is that we do not know the *BRCA* genetic status of studied patients. Thus, the likelihood that expression loss for *BRCA1* reflects genetic dysfunction in this gene is only assumption.

Women with BRCA1-associated BLBC have been found to have a similar clinical course as compared to no mutation carriers [28, 30]. In our series there was similar result, showing unfavorable prognostic value of the combination of the basal-like subtype of BC and a absent IHC expression of BRCA1 protein. Both were associated with <5-year survival of patients.

The immune system (IS) is important for the outcome of BC disease, but its relationship to tumor development and progression is complex and influenced by genetic, tumor-specific, and environmental factors. It is a dynamic process and depends on the inhibition and activation of signals forming a pro- or antitumor environment, reflected in a

different amount and variety of TILs, with possible participation of inhibitory pathways (e.g. associated with PD-L1).

The modulation of the IR, e.g. through immune checkpoint inhibitors or some chemotherapeutics (e.g. anthracyclines), facilitates the so-called "immunogenic cell death" and has a possible effect on highly mutated/genomically unstable tumors, e.g. basal-like BC [7, 26]. The optimization of predictive biomarkers for response to immunotherapy continues. Germinative mutations in the *BRCA1* and *BRCA2* genes associated with defective homologous DNA repair lead to pronounced carcinoma antigen presentation, with the formation of multiple carcinoma-specific antigens activating IS with pronounced IR. This makes the *BRCA1* and *BRCA2* mutated BC a subtype, in which immune modulation and immunotherapy, would have a beneficial effect [7, 26]. TILs are thought to be a possible prognostic factor in *BRCA* mutated BC, and a high TILs count may be predicting for positive BRCA status [26]. Determination of additional immune factors, incl. TILs subtypes and the expression of checkpoint molecules may help to clarify the role of IR in basal-like and TNBC, incl. with impaired BRCA function.

In our study, a comparative analysis of PD-L1 expression and cancer immune cell infiltrate according to BRCA1 expression showed no statistically significant differences. However we found that there is an activation of the immune response in BLBC subtype, including TNBC, confirmed by the higher levels of tumor-infiltrating cytotoxic CD8(+) T-lymphocytes and PD-L1-positive immune cells, infiltrating the tumor stroma. It is still unknown whether the mutation rate of breast tumor cells contributes to specific differences in the tumor infiltration of immune cells and PD-L1 expression [31]. We did not find data on the simultaneous study of PD-L1, lymphocyte subtypes and BRCA status, using the IHC method.

Treatment in cases of BC is still a problem, especially in the TN subtype, in which there is no *HER2*-targeted or endocrine therapy. Patients with the same therapy have different responses due to the heterogeneous molecular and genomic nature of this neoplasm [1, 7, 8, 17, 23]. Despite advances in the study of tumor characteristics, there are a small number of approved prognostic and predictive markers for treatment choice in patients with TNBC. Ensuring the most effective therapy by finding new predictive markers for therapeutic response is of paramount importance in the implementation of personalized medicine in these cases [22, 23, 25].

It is essential to understand the importance of *BRCA1/BRCA2* genetic dysfunction in BC, and some molecular characteristics may affect sensitivity to chemotherapy and DNA-damaging agents in these patients. Cases with TN, *BRCA*-mutated BC have been suggested to be more sensitive to chemotherapy than high grade TNBC without the *BRCA1/BRCA2* mutation [1, 17, 22, 27]. According to some studies, *BRCA*-mutated BC, incl. basal-like subtype, show higher sensitivity to DNA-damaging agents, for example platinum-containing (e.g., cisplatin) and poly (ADP ribose) polymerase (PARP) inhibitors. PARP inhibitors have an established effect in patients with metastatic HER2-negative BC with germline *BRCA1* and *BRCA2* mutations, but whether they are effective in those with acquired somatic *BRCA1* and *BRCA2* mutations or the BRCA-ness phenotype is not entirely clear. Some epigenetic mechanisms, mainly acquired *BRCA1* methylation, have been suggested to be a promising predictor for response to PARP inhibitor therapy in sporadic cases of BC [23]. Various mechanisms lead to primary resistance to platinum and PARP inhibitors, some of which are associated with inherited mutations in the *BRCA1* gene. During treatment, secondary mutations in the *BRCA* genes can lead to acquired resistance to therapy, and others to the recovery of their activity and the expression of the proteins encoded by them [20, 25].

Therefore, determining the status of *BRCA* allows the identification of some genetic and epigenetic disorders with probable prognostic and predictive therapeutic value in sporadic and familial cases of BC [20–22]. Finding test(s) that is safe, quick to implement and easily accessible to patients is essential.

There are currently some clear criteria for conducting genetic counseling and testing for *BRCA1/BRCA2* status in patients with BC [32–35]. It is recommended mainly in patients with some personal and family history (e.g. cancer diagnosed at age ≤45 years old, the presence of a neoplastic process in both breasts, diagnosed at age ≤60 years old with TNBC, the presence of the disease in at least two first-line relatives, a first- or second-line relative who has BC younger than 50 years old, male and second breast cancer, regardless of familial history and age at diagnosis etc.). The establishment of morphological, immunohistochemical and molecular characteristics suggesting alterations in the *BRCA1* and *BRCA2* genes may assist in the selection of patients suitable for genetic testing. The pathologist should suggested genetic counseling in the histological response due to the possibility of carrying a *BRCA1/BRCA2* mutation [1, 21, 25].

When selecting for genetic analysis, not only familial but also sporadic cases of BC should be keep in mind, because identification of some alterations in the *BRCA1* and *BRCA2* genes may allow more precise clinical and therapeutic behavior in these patients [20–22]. Clarification of *BRCA1/BRCA2* status and screening for specific mutations is no less prognostic for close relatives in the family of patients with BC, due to the possibility of detecting healthy individuals, but with a high risk of developing some neoplasms, including BC/OC and others [1, 20].

There is a wide variety of molecular-genetic tests to determine *BRCA* carrier, but they are expensive and time consuming to obtain a result due to the large size of the genes studied, the presence of hundreds of different mutations, including those without proven clinical significance, the lack of hot-spot regions with mutations to study. This requires a more precise selection of applicant families for mutation testing [1, 7].

Histopathological features, together with clinical data, can be used as a predictive factor for determining *BRCA1/BRCA2* status by mutation screening. Validation of IHC results using molecular confirmation may allow IHC also to facilitate the selection of high-risk cases suitable for genetic analysis [8]. An IHC analysis, which to determine the expression of BRCA-linked proteins that reflect impaired gene function, is a promising quick, low cost and easy to implement test.

The established contradictory data regarding the prognostic role of BRCA status in hereditary or sporadic cases with BC require further studies to clarify it. Finding correlations between clinico-pathological (morphological and IHC) and molecular characteristics of BRCA tumors can give a clearer picture of their biological behavior. This may allow development of a prognostic algorithm in patients with BC, which is important for more accurate determination of the clinical and therapeutic approach in them [1, 8, 18, 22, 36].

**Conclusions**

Our results show that there is a difference in the expression of BRCA1-protein in tumor cells in different surrogate molecular subtypes of BC, and it is most significant in the basal-like subtypes, which is more often with the TN phenotype. Using immunohistochemistry, it is possible to detect a clinically relevant type of protein expression that may reflect altered *BRCA1* gene activity, allowing better selection of patients for subsequent molecular genetic

analysis. More studies are needed to confirm the clinically meaningful applicability of IHC expression for BRCA in BC.

The phenotype of basal-like breast cancer with absent BRCA1 protein expression and higher rate of TILs may identify a group of patients, who may be subjected to genetic screening for the search of pathological mutations in *BRCA*. Further research and prospective validation are necessary to confirm our hypothesis.

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**Conflict of interest:** none declared

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**Table I.** Percentage distribution of clinico-pathological data in all studied patients and in different subtypes of BC

| Variables | LumA | LumB | HER2 | TN | All patients |
|---|---|---|---|---|---|
| | % | % | % | % | % |
| **age (yr)** | | | | | |
| ≤50 | 24.0 | 20.0 | 16.0 | 4.0 | 16.0 |
| >50 | 76.0 | 80.0 | 84.0 | 96.0 | 84.0 |
| **5 years** | | | | | |
| no | 8.0 | 36.0 | 72.0 | 64.0 | 45.0 |
| yes | 92.0 | 64.0 | 28.0 | 36.0 | 55.0 |
| **grade** | | | | | |
| G1 | 32.0 | 8.0 | 4.0 | 0.0 | 11.0 |
| G2 | 68.0 | 44.0 | 24.0 | 28.0 | 41.0 |
| G3 | 0.0 | 48.0 | 72.0 | 72.0 | 48.0 |
| **stage** | | | | | |
| I | 36.0 | 12.0 | 8.0 | 8.0 | 16.0 |
| II | 48.0 | 56.0 | 56.0 | 64.0 | 56.0 |
| III | 16.0 | 32.0 | 32.0 | 28.0 | 27.0 |
| IV | 0.0 | 0.0 | 4.0 | 0.0 | 1.0 |

| | | | | | |
|---|---|---|---|---|---|
| **metastatic lymph nodes** | | | | | |
| no | 76.0 | 36.0 | 36.0 | 64.0 | 53.0 |
| yes | 24.0 | 64.0 | 64.0 | 36.0 | 47.0 |
| **LVI** | | | | | |
| no | 96.0 | 72.0 | 60.0 | 76.0 | 76.0 |
| yes | 4.0 | 28.0 | 40.0 | 24.0 | 24.0 |
| **tumor size** | | | | | |
| ≤3 cm | 36.0 | 12.0 | 12.0 | 24.0 | 21.0 |
| >3 cm | 64.0 | 88.0 | 88.0 | 76.0 | 79.0 |
| **samples** | | | | | |
| excision biopsy | 28.0 | 12.0 | 24.0 | 24.0 | 22.0 |
| mastectomy | 72.0 | 88.0 | 76.0 | 76.0 | 78.0 |
| **histological type** | | | | | |
| NST | 68.0 | 80.0 | 92.0 | 80.0 | 80.0 |
| lobular carcinoma | 12.0 | 20.0 | 4.0 | 0.0 | 9.0 |
| other special type | 20.0 | 0.0 | 4.0 | 20.0 | 11.0 |
| **basal-like subtype** | | | | | |
| no | 96.0 | 92.0 | 88.0 | 52.0 | 82.0 |
| yes | 4.0 | 8.0 | 12.0 | 48.0 | 18.0 |

**Table II.** Comparative analysis of 5-year survival according to basal/non-basal-like BC (all patients)

| **Indicator** | **Non-basal-like** | | **Basal-like** | | **p** |
|---|---|---|---|---|---|
| | n | % | n | % | |
| **5-year survival** | | | | | 0.017 |
| no | 32 | 39.0 | 13 | 72.2 | |
| yes | 50 | 61.0 | 5 | 27.8 | |

**Table III.** Comparative analysis of 5-year survival according to BRCA1 expression (all patients)

| **Indicator** | **Negative** | **Positive** | **p** |
|---|---|---|---|

| | n | % | n | % | |
|---|---|---|---|---|---|
| **5-year survival** | | | | | 0.001 |
| no | 19 | 73.1 | 26 | 35.1 | |
| yes | 7 | 26.9 | 48 | 64.9 | |

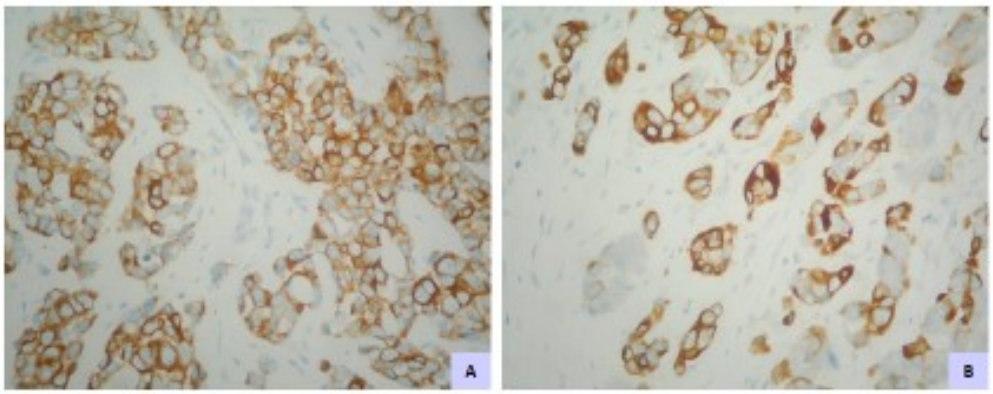


**Figure 1.** IHC expression model of CK5/6 (A) and CK17 (B) in basal-like TNBC (×400)

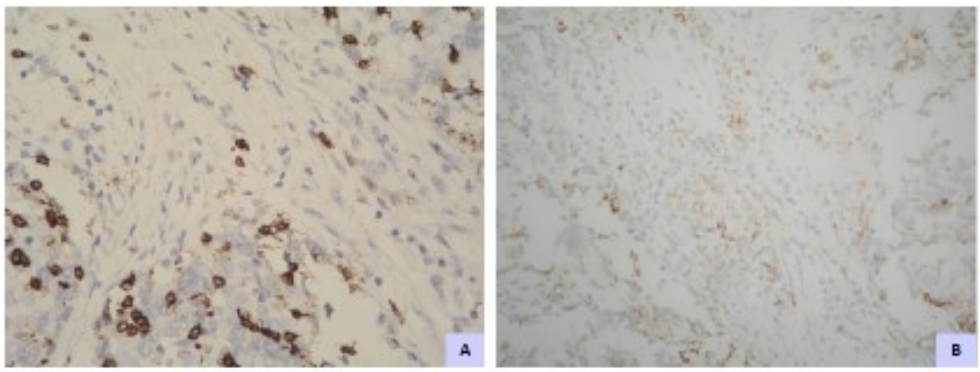


**Figure 2.** IHC staining for CD8 (A) and PD-L1 (B) in basal-like TNBC (×400)

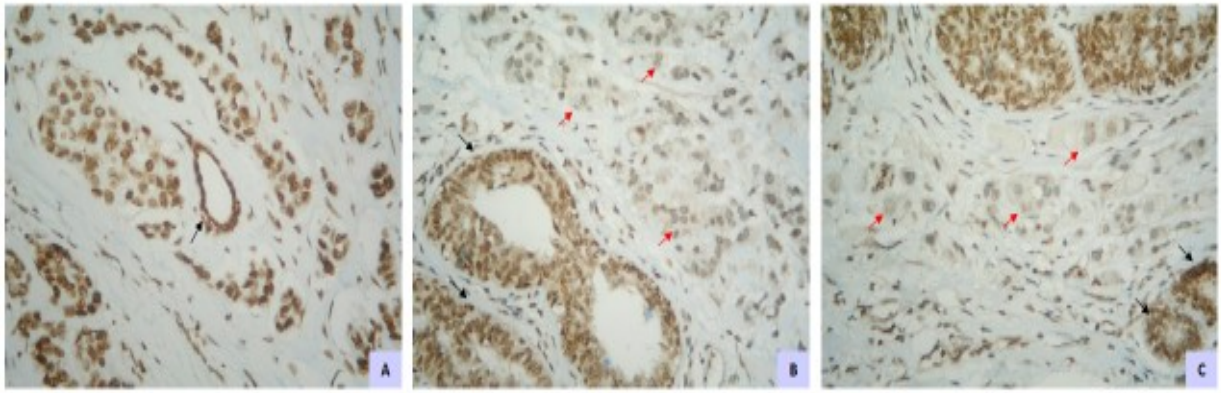


**Figure 3.** IHC staining for BRCA1 protein in BL TNBC – positive and negative expression in normal epithelial cells of breast (black arrow) and tumor cells of BC (red arrow), respectively (HPF ×400)