Assessment of programmed death-ligand 1 receptor immunohistochemical expression and its association with tumor-infiltrating lymphocytes and p53 status in triple-negative breast cancer

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Abstract
Breast cancer (BC) is the second most frequent type of cancer for both sexes combined, after lung cancer. Triple-negative BC (TNBC) molecular subtype is characterized by lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) immunoexpression or amplification and represent 10–20% of all BC cases. The issue of the present study was to analyze the associations between programmed death-ligand 1 (PD-L1) immunoexpression and distribution of stromal tumor-infiltrating lymphocytes (stTILs) combined with clinico-morphological features of patients with TNBC. Secondly, our research evaluated PD-L1 immunoexpression as a prognostic factor and its correlation with p53 immunoexpression. Thirty cases with primary TNBC without prior neoadjuvant therapy were included in this research. stTILs were identified in all cases, most of them with low distribution (66.7%). A positive immunoexpression for PD-L1 was observed in 40% of cases. The PD-L1 immunoexpression was statistically significant associated with age, pathological tumor size, lymphovascular invasion, stTILs level, the presence of cluster of differentiation 8-positive (CD8+) TILs and p53 immunoexpression. In the present study, a positive PD-L1 immunoexpression was associated with a worse distant metastasis free survival (DMFS). We also found not only that high stTILs level were associated with a better DMFS but also that there was a statistically significant association between stTILs level and PD-L1 immunoexpression. Our results bring new insights to the fine connections between tumor microenvironment and molecular changes of TNBC. It helps us to better understand these aggressive tumors to identify the more useful biomarkers for predicting the response to adjuvant therapy and can represent a method for selecting the most suitable patients for immunotherapy.

Keywords: triple-negative breast cancer, immunohistochemistry, PD-L1, tumor-infiltrating lymphocytes, p53.

Introduction
Breast cancer (BC) is the second most frequent type of cancer for both sexes combined, after lung cancer, with a 16.6% incidence worldwide, according to Bray et al. (2018) [1]. In the last two decades, numerous studies highlighted the huge heterogeneity of the BC and tailor therapies were developed to improve the survival and quality of life for these patients. Despite of all efforts, BC continues to be the leading cause of death in female population in more than 100 countries, with 15% mortality rate worldwide [1]. From the first molecular classification of BC realized by Perou et al. in 2000 [2] and Sørlie et al. in 2001 [3], huge progresses have been done in understanding the molecular biology of different morphological subtypes of BC and four molecular surrogate subtypes of BC emerged at the St. Gallen Consensus in 2013, with different therapeutic and prognostic implications: luminal A-like, luminal B-like, human epidermal growth factor receptor 2 (HER2)-positive and triple-negative BC (TNBC). Their definition is based on the immunoexpression of estrogen receptor (ER) and progesterone receptor (PR), proliferation rate and immunoexpression or amplification of HER2 [4].

TNBC subtype is characterized by lack of ER, PR and HER2 immunoexpression or amplification and represents 10–20% of all BC cases [5]. But this category of BCs is not a simple one and, in fact, it is extremely heterogeneous. It includes different morphological subtypes of BC, and it is associated with variable prognosis. Most of them have an aggressive clinical behavior comparing with the other subtypes. The heterogeneity of TNBC is under the debate of several studies, which try to characterize it furthermore, to unravel new potential therapeutic agents.
which can improve the overall survival (OS). A special attention is paid to immune microenvironment of the tumors and tumor-infiltrating lymphocytes (TILs) emerge as a biomarker for the immunogenicity of BC. High value of TILs is associated with an improvement of disease-free survival (DFS) and OS in TNBC, and it can be used as a strong prognostic factor [6–8]. The composition of TILs is complex, 75% of TILs are represented by T-lymphocytes, most of them being cluster of differentiation (CD)8+ (cytotoxic T-lymphocytes) and secondly CD4+ (T-helper cells) [9].

One way of the malignant cell to escape from immune defense is through the axis of programmed death 1 (PD1) receptor and its ligand [programmed death-ligand 1 (PD-L1)]. PD1 receptor, known also as “checkpoint molecule”, is expressed mainly on the mononuclear inflammatory cells and its activation by PD-L1 or PD-L2 ligands acts as a “brake” for the immune response [10]. Therapy with immune checkpoint inhibitors (ICIs) not only proved its efficacy in several aggressive cancers, like melanoma or non-small cell lung carcinoma, but recently is considered as a therapeutic option for metastatic or locally advanced TNBC [11].

**Aim**

The aim of the present study was to analyze the associations between PD-L1 immunoexpression and distribution of stromal TILs (stTILs) in conjunction with clinico-morphological features of patients with TNBC from a single institution in southeastern of Romania. The second objective was to evaluate the value of PD-L1 immunoexpression as a prognostic factor and its correlation with p53 immunoexpression.

**Patients, Materials and Methods**

**Patient cohort**

In the present study, cases were selected from the recoded medical data and electronic database of the Department of Clinical Pathology between 2014 and 2018. We identified all the patients clinically diagnosed with BC in the Department of Surgery of our Hospital, which proved to be ER/PR negative and with a HER2/neu negative status evaluated by immunohistochemistry (IHC) or/and by chromogenic in situ hybridization (CISH). All these cases were reviewed according to internal protocol of the Department of Clinical Pathology based on criteria of World Health Organization (WHO) breast tumor [12] and recommendations of St. Gallen Consensus, to establish molecular surrogate subtypes [4]. Thirty cases with primary TNBC without prior neoadjuvant therapy were included in this research. Clinico-morphological features were taken from pathological file of the patients including: age at the time of diagnosis, type of surgery, tumor size, morphological type of BC, tumor grade, the presence of in situ component, lymphovascular invasion, lymph node status, presence or absence of distant metastasis and type of adjuvant therapy followed by the patient after surgical intervention. It has been also recorded the date when distant metastases had been identified and the last date of follow-up, both criteria being useful for survival analysis. Incomplete clinical data or preoperative neoadjuvant therapy were considered as exclusion criteria. A written consent signed by each patient included in our study was available.

**TILs evaluation**

TILs evaluation was performed on usual Hematoxylin–Eosin (HE) whole stained slides according to the Guidelines established by the International TILs Working Group 2014 [13] by a trained pathologist on scoring TILs using the available on-line resource [13, 14]. A semiquantitative analysis of stTILs was performed as a continuous parameter (increment of 10%). The percentage of stTILs was defined by the average area occupied by all mononuclear inflammatory cell over total intra-tumoral connective tissue stroma, which was considered the adjacent area to invasive tumor [13]. For statistical analyses, it was further stratified in categorical variable in which absent or focal stTILs if <10%, low stTILs if the score was ≥10% – <50% and high stTILs if ≥50% [15].

**Immunohistochemical staining and evaluation**

The most representative formalin-fixed and paraffin-embedded blocks were sectioned 4 μm thick and followed the immunostaining protocol for manual method. Incubation of slides was done using the following ready-to-use, primary antibodies from Master Diagnóstica (Granada, Spain): rabbit anti-human PD-L1 monoclonal antibody (clone CAL10); rabbit anti-human p53 monoclonal antibody (clone SP5); rabbit anti-human CD8 monoclonal antibody (clone SP16). Master Polymer Plus Detection System, which include 3,3’-Diaminobenzidine (DAB) chromogen, was used for detection and brown staining of antigen concerned. The final step consisted in counterstaining with Mayer’s Hematoxylin and mounting the slides. Tonsil was used as positive control for PD-L1 and CD8 biomarkers and serous carcinoma of the ovary as positive control for p53 assessment. HER2 status was reviewed using the recommendations of American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) 2018 [16].

**Statistical analysis**

In the current research, Statistical Package for the Social Sciences version 20.0 (SPSS Inc.; Chicago, IL, USA) software was used for data recording and statistical analysis. Each data was labeled as either nominal or quantitative variable. A descriptive statistic was performed for quantitative variables. Comparisons of ratios in independent groups were performed with χ² (chi-squared) test. Kaplan–Meier method was applied to obtain the cumulative percentages of distant metastasis free survival (DMFS) time followed by log-rank tank test for further analyze. DMFS was defined as the time from initial diagnose to the time of distant metastasis. Those cases without distant metastases or those cases in which the follow-up had been lost before the established period of two years, were labeled as censored. A two-tailed p-value < 0.05 was considered significant.

**Results**

Thirty female patients were included in current study, with a mean age of 62.47±11.649 years (from 43 to 84 years), from which 73.3% were more than 50 years old. Most of the patients underwent radical mastectomies ±
regional lymphadenectomy, with a mean tumor diameter 42±34.482 mm (10–180 mm) and 16.7% of them were multifocal tumors. Regarding the morphological type, we observed a high prevalence for invasive ductal carcinoma–not otherwise specified (IDC-NOS) in our group (80%) and the remaining were represented by three cases of IDC with medullary features (Figure 1, A–D), one case of IDC with sebaceous differentiation, and one case with inflammatory BC. High histopathological grade was recorded in 63.4% of our cohort, most of them (75%) having also a positive reaction for PD-L1 (Figure 2). An intraductal component was observed in 66.7% of cases and lymphovascular invasion was noted within 15 cases. stTILs were identified in all cases, with mean 44±22.984, most of them with low distribution (66.7% of cases). According to the American Joint Committee on Cancer (AJCC) tumor stages, we observed that pT2 was the most frequent one (40%) followed by pT3 and pT4, each with six cases. The mean time of follow-up was 16.2±7.286 months (range 2–24 months).

We analyzed the pattern of distribution and the percentage of positive cells for each antibody. A positive immunoreaction for PD-L1 was considered when >1% of mononuclear inflammatory cells from the stromal compartment or/tumor cells are membranous stained [17] and it was observed in 40% of cases, from which 58.3% were positive in only stTILs (Figure 1B) and 16.7% positive only in tumor cells. A simultaneous positive immunostaining of both compartments was identified in 11 cases (Figure 2B).

Associations between PD-L1 immunoeexpression and the main clinico-pathological and IHC features of the cases included in our study are detailed in Table 1. The most statistically significant associations were represented by age more than 50 years old \((p=0.018)\), pathological tumor size \((p=0.025)\), lymphovascular invasion \((p=0.025)\), stTILs level \((p=0.018)\), the presence of CD8+ TILs \((p=0.004)\), and p53 immunoexpression \((p=0.044)\) (Table 1).
Table 1 – Clinico-pathological features of TNBC according to PD-L1 immunoreexpression

| Clinico-pathological features | PD-L1 negative n (%) | PD-L1 positive n (%) | $\chi^2$ score | p-value* |
|------------------------------|----------------------|----------------------|----------------|----------|
| Total cases                  | 18 (60.0%)           | 12 (40.0%)           |                |          |
| Age (years)                  |                      |                      |                |          |
| ≤50                          | 2 (11.1%)            | 6 (50.0%)            |                |          |
| >50                          | 16 (88.9%)           | 6 (50.0%)            |                |          |
| Morphological type           |                      |                      |                |          |
| IDC–NOS                      | 14 (77.8%)           | 10 (83.3%)           |                |          |
| Others                       | 4 (22.2%)            | 2 (16.7%)            |                |          |
| Tumor grade (G)              |                      |                      |                |          |
| ≥2                           | 8 (44.4%)            | 3 (25.0%)            |                |          |
| ≥3                           | 10 (55.6%)           | 9 (75.0%)            |                |          |
| DCIS                         |                      |                      |                |          |
| Yes                          | 13 (72.2%)           | 7 (58.3%)            |                |          |
| No                           | 5 (27.8%)            | 5 (41.7%)            |                |          |
| Pathological tumor size (pT) |                      |                      |                |          |
| pT1–pT2                     | 12 (66.7%)           | 3 (25.0%)            |                |          |
| pT3–pT4                     | 6 (33.3%)            | 9 (75.0%)            |                |          |
| Pathological lymph node status (pN) |              |                      |                |          |
| <3                          | 10 (55.6%)           | 3 (25.0%)            |                |          |
| ≥3                          | 8 (44.4%)            | 9 (75.0%)            |                |          |
| Lymphovascular invasion     |                      |                      |                |          |
| No                          | 12 (66.7%)           | 3 (25.0%)            |                |          |
| Yes                         | 6 (33.3%)            | 9 (75.0%)            |                |          |
| stTILs level                |                      |                      |                |          |
| <50%                        | 9 (50.0%)            | 11 (91.7%)           |                |          |
| ≥50%                        | 9 (50.0%)            | 1 (8.3%)             |                |          |
| CD8+ TILs                   | 8.167                | 0.004                |                |          |
| Negative (CD8– TILs)        | 4 (22.2%)            | 9 (75.0%)            |                |          |
| Positive (CD8+ TILs)        | 14 (77.8%)           | 3 (25.0%)            |                |          |
| Ki67 status                 | 1.118                | 0.290                |                |          |
| Low Ki67 index              | 3 (16.7%)            | 4 (33.4%)            |                |          |
| High Ki67 index             | 15 (83.3%)           | 8 (66.7%)            |                |          |
| p53 immunoreexpression      | 4.043                | 0.044                |                |          |
| p53 nonmutated              | 14 (73.7%)           | 5 (26.3%)            |                |          |
| p53 mutated                 | 4 (36.4%)            | 7 (63.6%)            |                |          |
| Metastatic relapse           | 15.648               | <0.001               |                |          |
| No                          | 16 (88.9%)           | 2 (16.7%)            |                |          |
| Yes                         | 2 (11.1%)            | 10 (83.3%)           |                |          |

CD8: Cluster of differentiation 8; DCIS: Ductal carcinoma in situ; IDC–NOS: Invasive ductal carcinoma—not otherwise specified; n: No. of cases; PD-L1: Programmed death-ligand 1; stTILs: Stromal tumor-infiltrating lymphocytes; TNBC: Triple-negative breast cancer. *Chi-squared test.

A membranous staining of the stTILs in more than 10% of cases was considered the cutoff for a positive immunoreaction to CD8 biomarker [18] and it was recorded for 56.7% of all cases (CD8+ TILs) (Figures 1C and 2C). It was observed only three cases with CD8+ TILs from those who had also a positive immunostaining for PD-L1, but a higher rate (77.8%) was noticed in the PD-L1 negative category. A positive immunostaining for p53 was consider when the nuclei were brown-stained, and 10% value was considered as the cut-off point [19]. The intensity and distribution of p53 immunostaining was also analyzed: “null-type” – no nuclear staining; “wild-type” – weakly and focal brown nuclear staining; “overexpression” – strong and diffuse nuclear staining [20]. An aberrant p53 protein immunoreactivity (“null-type” and “overexpression” type) was observed in 36.7% of cases, from which 63.6% were “overexpression” type (Figure 2D). The vast majority of those cases with an “overexpression” pattern (85.7%) were noticed in the CD8+ TILs category. Ki67 index was reassessed using criteria of the St. Gallen Consensus 2015, in which a 20% value was recommended as a cut-off value for low/high level of proliferate tumoral rate [21]. Median value for Ki67 index was 60, interquartile range (IQR) (30–80), most of the tumors having a high proliferation rate (76.7%). Most of the tumors with an immunopositive reaction for PD-L1 were associated with a high Ki67 index (66.7%).

PD-L1 immunoreexpression proved to have a significant influence on DMFS, analyzed by Kaplan–Meier method and log-rank test, with $p$<0.0001 [PD-L1 negative: mean 22.471, 95% confidence interval (CI): 20.473–24.468; PD-L1 positive: mean 11.375, 95% CI: 7.788–14.962] (Figure 3A). A statistically significant differences regarding DMFS was also observed for stTILs values with an improvement for high values of stTILs, $p$=0.014 (low stTILs: mean 15.359, 95% CI: 11.872–18.846; high stTILs: mean 22.800, 95% CI: 20.569–25.031) (Figure 3B). A CD8+ TILs also had a statistically significant positive effect on MDFS with $p$<0.001 (CD8– TILs: mean 12.215, 95% CI: 8.247–16.184; CD8+ TILs: mean 21.765, 95% CI: 19.462–24.068) (Figure 3C). The p53 status (mutational and non-mutational) has also an impact on DMFS with $p$=0.010 (p53 non-mutated: mean 20.659, 95% CI: 17.702–23.617; p53 mutated: mean 13.727, 95% CI: 9.495–17.960) (Figure 3D).

**Discussions**

TNBC is a relatively rare type of molecular subtype of BC, which is associated with a poor prognosis. Its morphological features and molecular changes make it difficult to treat, being responsible for up to 25% of BC deaths [22]. Because it is not responsive to hormonotherapy or to the Trastuzumab, chemotherapy and radiotherapy are the only accepted possibilities as an adjuvant therapy for these patients.

Immunotherapy is a new approach to treat tumors which are highly “immunogenic” (tumors who have the capacity to induce host adaptive immunity). ICIs can have a negative impact on tumor progression and huge successes were obtained when this new form of therapy was approved and administrated in a variety of malignancy, resulting in great improvements of DFS and OS [23, 24]. Starting from this point, which it was considered a “breakthrough in the year 2013” [25], a special attention was paid to the distribution and quantity of immune infiltrate through different morphological subtype of BC. In 2019, a big step was done by obtaining the approval of Atezolizumab (an ICI drug which target the PD-L1) as adjuvant therapy for advance or metastatic TNBC, secondary to the results obtained by IMpassion130, a phase III trial [11, 26]. Nevertheless, less than half of patients will benefit from immunotherapy [27] and further studies are necessary to refine those biological factors which can be used as predictive and prognostic factors.
In our study, we identified 40.0% of cases positive for PD-L1 in more than 1% of either tumor cells or stromal inflammatory cells. Differences between PD-L1 immunexpression and clinico-morphological features were analyzed, and we observed a statistically significant differences with age (≤50 years old), a positive PD-L1 immunexpression being associated with less than 50 years old. In the research of Zeng et al. (2019), which included 132 cases of TNBC, it was also observed statistically significant association with age, but a PD-L1 immunopositive tumors were more frequent associated with more than 50 years old, and no association with menopausal status [22]. In concordance with literature, the most predominant morphological type was represented by IDC-NOS, with no statistically significant differences between PD-L1 positive and PD-L1 negative group [22]. The same result was observed for “in situ” component, which was identified in 58.3% of PD-L1 positive cases. All ductal carcinoma in situ (DCIS) had not HER2/neu immunexpression, having a perfect concordance with the invasive component, as it was previously reported [28]. Even if some researches proved to be an association between PD-L1 immunexpression and tumoral grade or tumor proliferation rate, our study showed no statistically significant differences between PD-L1 positive and PD-L1 negative group, both being characterized by high rates of poorly differentiated tumors and high Ki67 index. We recorded a positive correlation for tumor diameter, the presence of lymphovascular invasion and the present of metastasis in more than three loco-regional lymph nodes (Table 1), similar with other studies [29, 30].

In the present study, a positive PD-L1 immunexpression is associated with a worse DMFS. This result is in concordance with data from the literature which also emphasized the negative impact of PD-L1 immunexpression over the DMFS in previous untreated patients [22, 31, 32]. But not all the studies had the same results as there are reports which had shown a better survival if there is high (>10%) PD-L1 immunexpression on tumor cells [33, 34].
All these divergent results reported in the literature lead to the necessity to further explore the role of PD-L1 immunoexpression on larger cohort for a longer period of follow-up, taking in considerations all the factors which can influence the prognosis.

Because TNBC is characterized by a highly tumor mutational burden, this leads to an enhancement of immune response and consecutively an increase levels of stTILs compared to other molecular BC subtypes [35]. The prognostic value of stTILs in TNBC was highlighted by several studies and clinical phase III trials, like FinHer trial [36] or Eastern Cooperative Oncology Group (ECOG) 2197 + ECOG 1199 [37]. Quantification of stTILs level is nowadays included in the last edition of breast tumors classification from WHO 2019, as a useful prognostic biomarker [12]. An increase with 10% of stTILs is associated with 15% reduced risk of relapse and 17% reduced risk of death regardless of adjuvant therapy [15]. Even if there are reports which demonstrated that high stTILs levels are more common in TNBC, still their levels varied great among tumors belonging to this BC molecular subgroup. In the present research, 33.3% of the cases had high stTILs level, consistent with other reports which demonstrated an increased level of stTILs in previously untreated patients with TNBC [36]. Loi et al. (2017) proved in their study that stTILs can be used as a predictive biomarker since high levels of stTILs are associated with a good response after Pembrolizumab immunotherapy (a humanized antibody which blocks PD-1 located on lymphocytes), and it is associated with an improvement of the objective response rate [36]. We also found not only that high stTILs level are associated with a better DMFS but also that there is a statistically significant association between TILs level and PD-L1 immunoexpression. In the study of Tomioka et al. (2018), it was noticed that low stTILs and high PD-L1 immunoexpression cases are characterized by the poorest DFS and OS, and it was suggested that immunotherapy should be more effective for these category of patients [32]. For these reasons, it is better both biomarkers to be evaluated and to be used together in prediction of ICIs therapy.

Not only presence of stTILs is important but also its composition. The microenvironment of a tumor is complex and different types of inflammatory cells are part of it, playing specific role in the progression of the malignant cells. A special attention had been paid to interactions between cytotoxic T cells – CD8 positive (CD8+), regulatory T cells – CD4 positive (CD4+), and regulatory forkhead box protein P3 (FOXP3) T-cells positivity. In the systematic
overexpression of p53, 85.7% of them being also positive for adjuvant therapy. Study of Lee et al. [45] demonstrated that a positive immunoexpression of CD8+ TILs, confirming the importance of using CD8 as a biomarker in the stratification of patients suitable for immunotherapy.

The tumor protein p53 (TP53) gene plays an important role in carcinogenesis and its mutation is identified in almost 80% of TNBC cases [22, 41]. There have been identified two types of TP53 mutations, which leads to an aberrant p53 protein immunoeexpression: those which are involving the protein-encoding reading frame and have as result an absence of p53 protein immunoexpression (“null-type” mutations), and “missense” mutations with an “overexpression” for p53 protein [42]. Consistent with data from literature, we also demonstrated that an aberrant p53 immunoexpression has a prognostic value since its presence is associated with a worse DMFS. The mutant p53 protein acquired more functions than the “wild-type” p53 protein (normal immunoeexpression) and can trigger the immune system by expressing itself on the tumor cell surface through histocompatibility complex (neoantigen) [22, 43, 44]. It was also demonstrated that mutation of TP53 gene can lead to a poor reaction of CD8+ T-cells, which can affect the response to the immunotherapy [45]. In our cohort, we identified six cases with overexpression of p53, 85.7% of them being also positive for CD8 TILs, in concordance with the study of Lee et al. (2019), which demonstrated that “missense” mutations of TP53 gene can trigger a highly immunological response different from the “null-type” mutations of TP53 in TNBC. So, even TNBC are frequently associated with a good immunological response, still these patients can be further stratified by sTILs and p53 immunoexpression because these potential biomarkers can predict a better response to adjuvant therapy. Study of Lee et al. (2019) on 798 of TNBC cases highlighted the importance to identify those cases with aberrant p53 immunoexpression because it can also predict a good response to immunotherapy [44], and novel therapy targeting this pathway of carcinogenesis may developed [46].

### Conclusions

The present research analyzes the association between PD-L1 immunoeexpression with multiple clino-morphological features of TNBC, with the presence of sTILs and its composition, and with p53 immunoeexpression. Our results bring new insights of the fine connections between tumor microenvironment and molecular changes and help us to better understand these highly aggressive tumors to identify the more useful biomarkers for predicting the response to adjuvant therapy. Testing both PD-L1 status and other factors which are correlated with PD-L1 immunoeexpression can represent a method for selecting the most suitable patients for the treatment with ICIs, as monotherapy or in combination with other drugs. Our results can represent a solid base for further researches on larger group, to be able to establish the best biomarkers with predictive and prognostic role, and may lead to the outlining of a diagnostic and treatment protocol.

### Conflict of interests

The authors declare that they have no conflict of interests.

### Authors’ contributions

Mariana Deacu and Gabriela Izabela Bălăţescu equally contributed to the manuscript.

### Informed consent

The experiments were performed with prior informed consent (written) from each participant.

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