p53 Missense Mutation is Associated with Immune Cell PD-L1 Expression in Triple-Negative Breast Cancer

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ABSTRACT
The programmed death ligand 1 (PD-L1) is a pivotal biomarker of immunotherapy in triple-negative breast cancer (TNBC). TP53 is reported as a positive regulatory predictor of immune efficacy. The correlation of p53 expression or mutation and PD-L1 expression is explored. By immunohistochemistry, PD-L1 expression between p53 mutation (missense and nonsense) and wild type; p53 no-expression/loss vs. expression were compared. There was a significant association between p53 mutation, especially missense mutation with higher histological grade, and PD-L1 expression in immune cells (ICs). Both p53 missense mutation and PD-L1 expression may be potential targets for improving immunotherapy response in TNBC.

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Introduction
Triple-negative breast cancer (TNBC) refers to breast cancer with negative immunohistochemical staining results for the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER-2). TNBC accounts for 15–20% of all pathological types of breast cancer (1). Surgery combined with chemotherapy is a traditional TNBC treatment. Although some patients with TNBC show a partial pathologic complete response after neoadjuvant chemotherapy (NAC), the 5-year survival rate of TNBC patients is less than 30% (2) when spread and metastasis occur.

Immunotherapy has played an increasingly important role in the clinical management of TNBC (3). The programmed cell death protein 1 (PD-1)/programmed death ligand 1 (PD-L1) axis has been confirmed as a significant immune checkpoint, and high PD-L1 expression in tumor cells contributes to immune escape and poor prognosis. Therefore, patients with high PD-1 or PD-L1 expression may benefit from anti-PD-1/PD-L1 inhibitor (4). Recently, pembrolizumab (5) is approved in metastatic TNBC patients with PD-L1 expression. The detailed regulatory mechanism of PD-L1 expression in TNBC is currently unclear, and the factors closely related to the expression of PD-L1 are needed to be explored.

TP53 is a tumor suppressor gene that participates in the regulation of cell proliferation, cell cycle, and DNA damage repair (6). TP53 mutation is closely associated with tumorigenesis and progression. Recent study has showed that there is a correlation between p53 and PD-L1 expression. In melanoma, a higher frequency of PD-L1 positivity was observed in p53 mutated cells (7). In oral squamous cell carcinoma, the expression of p53 and PD-L1 in tumor cells showed an obvious positive correlation (8). TP53 is reported as a more commonly mutated gene; and its mutation rate is over 80% in TNBC (9). p53 mutation was usually determined by immunohistochemistry (IHC) using a TP53 mutated-type antibody.
All-or-none expression patterns of p53 hints mis-sense or nonsense mutation of TP53 gene. Whereas weak staining of p53 means TP53 gene is wild type. Thus, the expression or mutation of p53 could be determined by IHC. Whether p53 expression or mutation is associated with PD-L1 expression in TNBC is worth to be confirmed.

In this study, we investigated the association between PD-L1 protein expression with p53 mutation (nonsense and missense) or p53 expression in TNBC. We also provide theoretical evidence for the function of p53 mutation in patients with TNBC undergoing immunotherapy.

Materials and methods

Patients and tissue specimens

Samples were collected from 155 TNBC patients who underwent radical surgery between 1/2017 and 4/2020 in Shandong University Qilu Hospital. All these cases were not received NAC before surgery. Clinicopathological characteristics of all samples were available from pathology reports and medical records (See supplementary Table). Informed consent was obtained, and the study was approved by The Ethics Committee of Shandong University (KYLL-2019-2-010). The procedures involving human subjects were in accordance with the Declaration of Helsinki.

Immunohistochemistry

Full face sections (5 μm) from formalin-fixed paraffin-embedded blocks of 155 cases were used to measure the expression of ER, PR, HER-2, Ki67, p53, and PD-L1. Antibodies against ER (SP1, Ventana), PR (1E2, Ventana), HER-2/neu (4B5, Ventana), Ki67 (30-9, Ventana), p53 (DO-7, ZSGB-BIO), and PD-L1 (SP142, Abcam) were used for immunohistochemical evaluation. The staining was carried out on an automatic immunohistochemical staining device (Benchmark XT, Ventana Medical Systems, USA) according to standard procedures.

Immunostaining evaluation

ER and PR expressions were defined as negative if no cells or <1% tumor cells had nuclear immunostaining. The negative immunostaining of HER2 was defined as: 0, 1+ immunostaining, and 2+ immunostaining but fluorescence in situ hybridization (FISH) negative. Specifically, 0: No staining or ≤10% of infiltrating cancer cells present showed incomplete and weak cell membrane staining; 1+: >10% of infiltrating cancer cells present showed incomplete and weak cell membrane staining; 2+: >10% of infiltrating cancer cells showed incomplete and/or weak to moderate cell membrane staining; or ≤10% of infiltrating cancer cells showed strong and complete cell membrane staining. The cases with positive staining of ER or PR or HER2 3+ (≥10% of infiltrating cancer cells showed strong and complete cell membrane staining) and HER2 2+ but FISH positive were all excluded. The criterion of HER2 FISH is referred to the ASCO/CAP 2018 breast cancer HER2 testing guidelines (10). Ki67 low expression was defined as ≤14% tumor cells nucleus positive; and >14% tumor cells nucleus positive was defined as Ki67 high expression (11,12).

Greater than or equal to 1% stained tumor cells (TCs) or immune cells (ICs: lymphocytes, macrophages, dendritic cells, granulocytes and plasma cells) are defined as PD-L1 positive, whereas <1% stained TCs or ICs are defined as PD-L1 negative (13). The positive rate of TCs is a proportion of PD-L1-positive tumor cells to all tumor cells. The positive rate of ICs is a proportion of area which occupied by immune cells stained with PD-L1 at any intensity to tumor area. IC1 is defined as 1–5% of ICs with PD-L1 expression in around or within the tumor. 5–10% or ≥10% of ICs with PD-L1 expression in around or within the tumor are separately classified as IC2 and IC3.

The pattern of p53 expression in TNBC included mutation and wild types. Mutation showed two patterns: nonsense and missense. Tumor cell nuclei staining with variable and weak intensity (regardless of stained cell proportion) of p53 is defined as the wild-type pattern. Complete negative nuclei staining of tumor cells is defined as nonsense mutation and no-expression/loss of p53. Missense mutation is defined as ≥80% of tumor cell nuclei stained with diffuse strong intensity (14,15). Both wild and missense
mutation types are included as p53 expression group. All immunostaining assessments were determined by two pathologists.

**Tumor infiltrating lymphocytes (TILs) evaluation**

TILs were assessed in the boundaries of the invasive tumor (defined as the area centered between the normal tissue and the border of the cancer nest, with a range of 1 mm), which calculated as a percentage of the area infiltrated by mononuclear cells (including lymphocytes and plasma cells) of the tumor interstitial area, according to recommendations by an International TILs Working Group 2014 (16). A 10% cutoff was used to distinguish low vs high levels of TILs (17).

**Statistical analysis**

The association between expression of PD-L1 with p53 expression or mutation and pathological parameters were analyzed using the Chi-squared test or Fisher’s exact test by Prism software. All tests were two-sided, and statistical significance was set at $p < 0.05$.

**Results**

**PD-L1 expression in TNBC**

Immunohistochemistry was performed to evaluate PD-L1 expression in 155 cases of TNBC. PD-L1 expression was observed in TCs and ICS. However, PD-L1 staining was mainly observed in the ICs of TNBC. Negative PD-L1 expression was found in 103 cases (66.45%, Figure 1(A)), and positive PD-L1 expression was found in 52 cases (33.55%), including 49 cases with ICs (94.23%, Figure 1(B)) and 3 cases with both positive PD-L1 expression of TCs and ICs (5.77%; Figure 1(C)). Matched Hematoxylin eosin (HE) staining better shows the morphology of tumor and immune cells (Figure 1(D–F)). No significant

![PD-L1 expression in TNBC](image)

**Figure 1.** PD-L1 expression in TNBC. Negative expression of PD-L1 of one case is showed, with complete no staining in both TCs and ICs (A). PD-L1 positive expression of one case, shows negative expression in TCs but positive expression of ICs. No staining of PD-L1 in TCs, but positive cytoplasm staining of PD-L1 in ICs could be seen (B). PD-L1 positive expression of another case, shows positive staining in both TCs and ICs. A membrane staining in TCs and positive cytoplasm staining of ICs of PD-L1 is observed (C). Corresponding Hematoxylin-Eosin (HE) staining of the three cases are shown to better display the morphology of tumor and immune cells (D, E, F). The scale bar represents 500 pixels.
association was observed between PD-L1 expression and age, T stage, N stage, M stage, lymph node metastasis, clinical stage, lymphovascular invasion, TILs and Ki67 between the p53 mutation and wild-type groups \((p > 0.05; \text{Table 2})\). However, there was a significant difference in histological grade between the p53 mutation and wild-type groups \((p = 0.0345; \text{Table 2})\), which suggested that p53 mutation might be related to higher histological grade and poor prognosis. Additionally, no-expression of p53 was found in 66 cases (42.58%) and p53 expression was found in 89 cases (57.42%). There was no significant difference observed in age, T stage, N stage, M stage, lymph node metastasis, histological grade, clinical stage, lymphovascular invasion, TILs and Ki67 index between the p53 nonsense and p53 missense mutation groups, no-expression and expression groups \((p > 0.05; \text{Table 2})\).

**p53 Mutation indicates a poor prognosis**

By MSK database (Cancer cell 2018), we collected 207 cases of TNBC, including 180 cases with p53 mutation and 37 cases with p53 wildtype. The Kaplan–Meier analysis showed a worse overall survival in p53 mutation group, compared with p53 wild-type group [median months overall (95%CI) 69.20 (51.40–98.20) vs. 235.40 (118.40–NA); \(p < 0.001\); supplementary Figure]. This result indicated that p53 could be act as a prognostic factor.

**PD-L1 expression of ICs is associated with p53 missense mutation**

To confirm whether PD-L1 expression is associated with p53 mutation or expression, a comparative analysis was performed. Of the 103 cases with PD-L1 negative expression, there were 19 cases (18.44%) with p53 missense mutation, 48 cases (46.60%) with p53 nonsense mutation, and 36 cases (34.95%) with wild-type. In the PD-L1 positive expression group (52 cases total), there were 18 cases (34.62%) with p53 missense mutation, 18 cases (34.62%) with p53 nonsense mutation (Figure 2(B)) and 37 cases (35.92%) with nonsense mutation (Figure 2(C)).

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### Table 1. Clinical characteristics of patients according to PD-L1 expression.

|                  | PD-L1 negative \((N = 103)\) | PD-L1 positive \((N = 52)\) | \(p\) Value |
|------------------|-------------------------------|-------------------------------|-------------|
| Age              |                               |                               |             |
| \(\leq 40\)     | 14                            | 10                            | 0.3976      |
| \(41–60\)       | 59                            | 24                            |             |
| \(\geq 61\)     | 30                            | 18                            |             |
| T stage          |                               |                               |             |
| T1 (\(\leq 2\)) | 58                            | 23                            | 0.2338      |
| T2 (2–5)        | 41                            | 28                            |             |
| T3 (\(> 5\))   | 4                             | 1                             |             |
| N stage          |                               |                               |             |
| N0               | 62                            | 34                            |             |
| N1               | 24                            | 15                            |             |
| N2               | 6                             | 2                             |             |
| N3               | 11                            | 1                             |             |
| Lymph node metastasis |                     |                               | 0.6008      |
| Absent           | 62                            | 34                            |             |
| Present          | 41                            | 18                            |             |
| M stage          |                               |                               | 1.0000      |
| M0               | 102                           | 52                            |             |
| M1               | 1                             | 0                             |             |
| Clinical stage   |                               |                               | 0.1310      |
| I                | 36                            | 26                            |             |
| II               | 49                            | 23                            |             |
| III              | 17                            | 3                             |             |
| IV               | 1                             | 0                             |             |
| lymphovascular invasion |                     |                               | 1.0000      |
| Absent           | 56                            | 29                            |             |
| Present          | 47                            | 23                            |             |
| Histological grade |                             |                               | 0.2576      |
| II               | 31                            | 11                            |             |
| III              | 72                            | 41                            |             |
| Tumor infiltrating lymphocytes |                 |                               | 0.0418*     |
| High             | 43                            | 31                            |             |
| Low              | 60                            | 21                            |             |
| Ki67 index       |                               |                               | 0.0293*     |
| \(\leq 14\%\)  | 9                             | 0                             |             |
| >14%             | 94                            | 52                            |             |

*Significant difference; PD-L1: the programmed death ligand 1.
mutation, and 16 cases (30.77%) with wild-type. Totally, p53 mutation was identified in 67 cases (65.05%) in the PD-L1 negative group and 36 cases (34.95%) in the PD-L1 positive group, and p53 expression was identified in 55 cases (61.80%) in the PD-L1 negative group and 34 cases (38.20%) in PD-L1 positive group. PD-L1 expression was not statistically different between the p53 mutation and wild-type groups, as well as the p53 no-expression and expression groups. However, a statistically significant difference in PD-L1 expression was found between the p53 missense and nonsense groups ($p = 0.0337$; Table 3), demonstrating that PD-L1 positive expression might be closely related to p53 missense mutation.

**PD-L1 expression level of ICs is not related to p53 missense mutation**

IC expression level of PD-L1 was classified into three groups: IC1, IC2 and IC3. Of the 52 cases, there were 25 cases (48.07%) in the IC1 group (Figure 3(A)), 14 cases in the IC2 (26.93%, Figure 3(B)) group, and 13 cases (25%, Figure 3(C)) in the IC3 group. Matched HE stain were also showed separately (Figure 3(D–F)). Specifically, 18 cases (72%) had p53 mutations (including 9 cases (36%) with missense and 9 cases (36%) with nonsense mutation) and 7 cases (28%) were p53 wild type; 9 cases (36%) had no expression of p53 and 16 cases (64%) expressed p53 in the IC1 group. In the IC2 group, 9 cases (64.29%) had p53 mutations (including 5 cases (35.72%) with missense and 4 cases (28.57%) with nonsense mutation) and 5 cases (35.72%) were p53 wild type; 4 cases (28.57%) had no expression of p53, while 10 cases (71.43%) expressed p53. In the IC3 group, 9 cases (69.23%) had p53 mutations (including 4 cases (30.77%) with missense and 5 cases (38.46%) with nonsense mutation), and 4 cases (30.77%) were p53 wild type; 5 cases (38.46%) had no expression of p53, while 8 cases (61.54%)
expressed p53 (Table 3). No significant difference was observed between the IC1, IC2, and IC3 groups, regardless of p53 mutation or expression status ($p > 0.05$).

**Discussion**

Immunotherapy for TNBC has gained increasing attention from researchers in recent years. Based on gene expression profiles, studies have identified ‘immunomodulatory’ (IM) as a molecular subtype (18) of TNBC. Because the immune cell signaling pathways in this subtype are in an active state, these patients may benefit from immunotherapy and have a better prognosis compared to the basal-like immunosuppressive (BLIS) subtype (19). PD-L1 is an important biomarker, and its expression level is closely associated with the response to immunotherapy. The determination of PD-L1 expression and the

| Table 2. Clinical characteristics of patients according to p53 mutation and expression. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Nonsense (N = 66) | Missense (N = 37) | Wild type (N = 52) | Mutation vs. wild type | p Value | Missense vs. nonsense | No expression vs. nonsense |
| Age             | 2.2398           | 0.9033           | 0.4328           |                  |
| ≤40             | 10               | 5                | 9                |                  |
| 41–60           | 39               | 21               | 23               |                  |
| ≥61             | 17               | 11               | 20               |                  |
| T stage         |                  |                  |                  |                  |
| T1 (<2)         | 36               | 17               | 28               | 0.5675           | 0.1554 | 0.1607 |
| T2 (2–5)        | 26               | 20               | 23               |                  |
| T3 (≥5)         | 4                | 0                | 1                |                  |
| N stage         |                  |                  |                  |                  |
| N0              | 41               | 25               | 30               | 0.2944           | 0.8236 | 0.5186 |
| N1              | 16               | 7                | 16               | 0.3990           | 0.4099 | 0.2542 |
| N2              | 2                | 2                | 4                |                  |
| N3              | 7                | 3                | 2                |                  |
| Lymph node metastasis |            |                  |                  |                  |
| Absent          | 42               | 25               | 29               | 1.0000           | 1.0000 | 0.4258 |
| Present         | 24               | 12               | 23               |                  |
| M stage         |                  |                  |                  |                  |
| M0              | 65               | 37               | 52               | 0.7158           | 0.7447 | 0.4355 |
| M1              | 1                | 0                | 0                |                  |
| Clinical stage  |                  |                  |                  |                  |
| I               | 28               | 15               | 19               | 0.7158           | 0.7447 | 0.4355 |
| II              | 27               | 18               | 27               |                  |
| III             | 10               | 4                | 6                |                  |
| IV              | 1                | 0                | 0                |                  |
| Histological grade |              |                  |                  |                  |
| II              | 18               | 4                | 20               | 0.0345*          | 0.0799 | 1.0000 |
| III             | 48               | 33               | 32               | 0.2953           | 0.8299 | 0.7403 |
| Lymphovascular invasion |      |                  |                  |                  |
| Absent          | 40               | 19               | 26               | 0.8651           | 0.1155 | 0.1391 |
| Present         | 26               | 18               | 26               |                  |
| Tumor infiltrating lymphocytes | |                  |                  |                  |
| High            | 36               | 14               | 24               | 0.4848           | 0.6519 | 1.0000 |
| Low             | 30               | 23               | 28               |                  |
| Ki67 index      |                  |                  |                  |                  |
| ≤14%            | 4                | 1                | 4                | 0.1719           | 0.1913 | 0.1440 |
| >14%            | 62               | 36               | 48               |                  |

*Significant difference.

| Table 3. Association between p53 expression and mutation with PD-L1 expression of ICs. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| PD-L1 expression of ICs         | ICs             | IC1 (N = 25)    | IC2 (N = 14)    | IC3 (N = 13)    | p Value         | p Value         | p Value         |
| p53 Type                        | Wild            | 0.7191          |                  |                  | 0.8822          |                  | 0.8948          |
| Mutation                        | 67              | 36              | 18              | 9               | 9               | 4               |
| Wild                            | 36              | 16              | 7               | 5               | 4               |                 |
| p53 Mutation                    |                  | 0.0337*         |                  |                  | 0.8470          |                  | 0.8470          |
| Missense                        | 19              | 18              | 9               | 5               | 4               |                 |
| Nonsense                        | 48              | 18              | 9               | 4               | 5               |                 |
| p53 Expression                  |                  | 0.1719          |                  |                  | 0.8470          |                  | 0.8470          |
| No-expression                   | 48              | 18              | 9               | 4               | 5               |                 |
| Expression                      | 55              | 34              | 16              | 10              | 8               |                 |

*Significant difference; PD-L1: the programmed death ligand 1; ICs: immune cells.
factors affecting its expression has important clinical value.

In this study, we found that PD-L1 was mainly expressed in ICs but not TCs in TNBC. Of the 52 cases with PD-L1 positive expression, only 3 cases (5.77%) exhibited positive PD-L1 expression in the TCs. Moreover, positive PD-L1 expression in ICs was also observed in these 3 cases. In the Phase III Impassion 130 study, atezolizumab plus nab-paclitaxel showed clinical benefit in advanced/metastatic TNBC patients who were PD-L1 positive (ICs ≥ 1%) using the SP142 IHC assay (20). Thus, evaluation PD-L1 positive expression of ICs in TNBC might be clinically significant. Zhang et al. confirmed high PD-L1 expression was a prognostic indicator for reduced overall survival of breast cancer from 5 studies containing 2,546 cases (21). In this study, the cases with PD-L1 positive expression displayed a higher Ki67 proliferation index, also suggesting more aggressive biological behavior.

TP53 is an important suppressor gene that plays a key role in physiological processes. When the TP53 gene is mutated, it loses its regulatory effect on cell growth, apoptosis, and DNA damage repair due to its spatial conformation changes, which may lead to tumorigenesis (22). Kim et al. found a worse prognosis of patients with TP53 mutation and high expression in TNBC (23). By MSK database, the Kaplan–Meier analysis showed a reduced overall survival in p53 mutation group, compared with p53 wildtype group. Both results suggested that p53 mutation might be a potential predictive marker of TNBC. p53 mutants recently have become a new target for cancer therapy. For example, Synnott et al. found that COTI-2, a third-generation thiosemicarbazone, acts by reactivating mutant p53 to its wild form and inhibits tumor cell growth in TNBC (24).

Previously, the mutation of the TP53 gene has been detected by sequencing. However, p53 mutation, detected by IHC has been used in some tumors, because that TP53 gene mutation could lead to mutant protein accumulation in tumor cell nuclei. Until now, the prognostic significance of IHC-detected p53 protein in TNBC

Figure 3. The expression levels of PD-L1 in ICs. One case of IC1, about one percent positive staining of PD-L1 in ICs is showed (A). One case of IC2, 5% positive staining of PD-L1 in ICs is showed (B). Another case of IC3, 10% positive staining of PD-L1 in ICs is showed (C). Corresponding HE staining of the three cases are also showed (D, E, F). The scale bar represents 500 pixels.
remains controversial, perhaps due to different interpretation methods and cutoff values. In ovarian carcinoma, mutation status of p53 by IHC was almost 100% accurate compared with next generation sequencing (14). Singh et al. also found p53 expression by IHC (the accuracy was 92.3% and the sensitivity was 94.3%) was an accurate surrogate for TP53 mutation analysis in endometrial carcinoma (15). Strong diffuse staining of ≥80% of tumor cell nuclei was identified as p53 mutant/overexpression in these two articles. Moreover, compared to TP53 sequencing, p53 IHC is more economical and quicker. It could be used as a reliable and optional detection method presently. Li et al. showed that high p53 expression by IHC detected is a promising prognostic candidate for poor survival (25). In this study, we found that p53 mutation by IHC was associated with high histological grade, which cases also showed diffuse strong expression pattern of p53, compared with the p53 wild type.

PD-L1 expression and p53 mutation or expression (mutation vs. wild; missense vs. nonsense; and no expression/loss vs. expression) were compared and analyzed. The results showed there was no statistically significant difference in PD-L1 expression between the p53 mutation and wild-type groups, as well as the p53 no-expression/loss and expression groups. However, between the p53 missense and nonsense groups, there was a significant difference in PD-L1 expression. Therefore, we speculated that p53 mutation are critical to PD-L1 expression. A p53 missense mutation may lead to over-expression of PD-L1 in ICs. However, there was no significant difference in PD-L1 expression levels in ICs (IC1, IC2, and IC3) between p53 mutation and wild type, missense and nonsense, or the no-expression/loss and expression groups.

Studies have shown that the TNBC is more likely to benefit from immune checkpoint blockade therapy due to its higher enrichment by TILs. Such as, more TILs are found in or around the tumor stroma of the IM type of TNBC (18), and these lymphocytes are positive for CD8, indicating that they are highly immunogenic. Recently, the association between p53 mutation and the number of TILs in TNBC was reported (26). Miseon et al. (27) proposed a hypothesis that p53 mutation produces a mutated p53 protein, which acts as a neoantigen, potentially triggering the immune response and increasing TILs level. In this study, high TILs seem to be observed in the cases with PD-L1 positive expression. Furtherly, Li et al. found there were more CD4/CD8+ regulatory T cells in p53 mutation cases than wildtype in breast cancer (28). Treg (Regulatory CD4+ T cell) was found to be associated with a high mutation rate of TP53 genes in breast cancer, and Treg abundance may be a potential predictive biomarker of pathological complete response after NAC in TNBC (29).

However, there are some limitations in this study. The correlation of p53 mutation and TILs, evaluation of different types immune cells of TILs, and the detailed molecular mechanism of p53 mutation and TILs or T cells subsets needs to be further explored. Additionally, the detailed follow-up data of the samples included in this study was not available due to the short time. More cohort from different centers or survival analysis are needed to approve the correlation between p53 mutation and PD-L1 expression and the prognosis value of p53 mutation and PD-L1 expression.

To summarize, the results of this study showed that p53 missense mutation, but not nonsense or expression, was related to PD-L1 positive expression of ICs in TNBC. The role for p53 missense mutation in the regulatory expression of PD-L1 was also revealed. Both p53 missense and PD-L1 expression could be potential predictors for immunotherapy response in TNBC.

**Declaration of interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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**Data availability statement**

All the data used to support the findings of this study are available from the corresponding author upon request.
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