Tim-3, PD-1, CD244 and Foxp3 Positive T Cells' Relation to the Prognosis of Dermatomyositis and Polymyositis Patients

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ABSTRACT

Objective: To explore the frequency of circulating $CD4^+$ T cells expressing $PD\text{-}1^+$, $TIM\text{-}3^+$ in polymyositis (PM) and dermatomyositis (DM) patients and its correlation with inflammatory factors, $CD244^+$ and $FOXP3^+$ T cell subtypes and prognosis.

Study Design: Observational study.

Place and Duration of the Study: Ganzhou people's Hospital, Ganzhou, Jiangxi, China, from July 2019 to June 2021.

Methodology: PM and DM patients were treated according to the institution's guidelines and followed up for 2 years. Fifty healthy volunteers were enrolled as controls. Serum interleukin (IL)-6, C-reactive protein (CRP), IL-17, and tumour necrosis factor α (TNF-α) levels were detected by enzyme-linked immunosorbent assay (ELISA). $TIM\text{-}3^+$, $PD\text{-}1^+$, $CD244^+$, and $FOXP3^+$ expressions were measured using flow cytometry. Inability to live normally, recurrence or death was defined as poor prognosis.

Results: The ESR, ALT, AST, LDH and ferritin concentration in PM/DM patients were remarkably elevated than that in healthy volunteers. The frequencies of $PD\text{-}1^+$, $TIM\text{-}3^+$, $CD244^+$, and $FOXP3^+$ were all remarkably enhanced in PM/DM patients compared with the healthy volunteers. The frequencies of $PD\text{-}1^+$, $TIM\text{-}3^+$, $FOXP3^+$, and $TIM\text{-}3^+/PD\text{-}1^+$ T cells were significantly elevated in the poor prognosis group compared with the good prognosis group. The frequency of $CD4^+TIM\text{-}3^+PD\text{-}1^+$ had satisfactory diagnostic value for PM/DM patients with bad prognoses. IL-17, $TIM\text{-}3^+$, $PD\text{-}1^+$and $TIM\text{-}3^+$ $PD\text{-}1^+$ were the risk factors for PM/DM patients with bad outcomes.

Conclusion: The frequency of circulating $CD4^+$ T cells expressing $TIM\text{-}3^+PD\text{-}1^+$ could be used to predict the prognosis of PM/DM patients.

Key Words: *Tim-3, PD-1, Dermatomyositis, Polymyositis, Inflammatory.*

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INTRODUCTION

As a rare autoimmune disease, dermatomyositis (DM), and polymyositis (PM) are the two main subtypes of idiopathic inflammatory myopathy (IIM).[1,2] The risk factors of PM/DM were still unclear. It has been reported that the pathogenesis of PM/DM is related to genetics, tumour, virus infection, abnormal immune mechanism, and other factors.[1] The typical pathological changes of PM/DM are inflammatory cell infiltration in skeletal muscle tissue, which not only damages muscle tissue, but also often involves other important organs such as the lung and heart.[3]

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In addition, PM/DM can even lead to secondary malignant tumours, which seriously influence the quality of life and prognosis of the patients.[4] However, the diagnosis of PM/DM currently relies on patient symptoms, signs, muscle biopsies, *etc*.[5] Without sensitive and reliable methods for early diagnosis. Therefore, it is important to clarify the pathogenesis of inflammatory myopathies, find serological and cytological tests that can be used for early diagnosis, and discover more precise and effective targeted therapeutic pathways to improve patient prognosis.

More than 80% of PM/DM patients have autoantibodies, demonstrating the adaptive immune system's role in the pathophysiology of PM/DM.[6] Large numbers of $CD4^+$ cells, $CD8^+$ T cells, and macrophage infiltration were also observed in muscle biopsies of PM and DM patients.[7] These T cell subtypes can also be found in the peripheral blood of PM and DM patients.[8] Previous studies have shown that T cell subtypes (CD244 and FOXP3, *etc*.) in muscle and peripheral blood of PM/DM patients are altered and can be used to assess disease activity and predict patient prognosis, but the number of such studies is still small.[9] Programmed

cell death protein 1 (PD-1) and T cell immunoglobulin mucin-3 (TIM-3) are important regulators of cell-induced immune responses, expressing on various immune cells.[10] In addition, co-expression of PD-1$^+$TIM-3$^+$ on cells has been reported to correlate with clinical outcomes in many autoimmune diseases such as rheumatoid arthritis (RA),[11] systemic lupus erythematosus (SLE),[12] and to predict patient prognosis. However, less relevant clinical studies have focused on the PD-1 and TIM-3 expressing on PM/DM T cells and the prediction of prognosis.

This study may reveal the clinical significance of PD-1 and TIM-3 in PM/DM patients, as well as provide novel research targets for PM/DM treatment. The aim of this study was to explore the frequency of circulating CD4$^+$ T cells expressing TIM-3$^+$, PD-1$^+$in PM/DM patients and its correlation with inflammatory factors, CD244$^+$ and FOXP3$^+$ T cell subtypes and patients' clinical outcomes.

METHODOLOGY

This prospective observational study recruited 44 PM and 38 DM patients who were admitted to the Ganzhou people's Hospital, from July 2019 to June 2021. PM/DM patients were diagnosed according to the diagnostic criteria for PM and DM by Bohan and Peter in 1975.[5] The inclusion criteria were the first episode and the patient did not receive immunosuppressive therapy. The exclusion criteria were age ≤18 years; patients with other autoimmune diseases; patients with serious infection, severe liver or renal dysfunctions, malignancy, and cardiovascular dysfunctions. In addition, the authors enrolled 50 healthy volunteers who came to the hospital for medical checkups as healthy group. All patients were routinely treated according to the institution's guidelines according to the patient's condition and followed up for 2 years. During the follow-up period, if the patient was unable to live normally (evaluated by the disability scale of the Health Assessment Questionnaire, MITAX and MTT8[13]), or if there was recurrence or death, it was defined as poor prognosis. This research has obtained approval from the ethics committee of the Ganzhou people's Hospital. All patients signed informed consent form.

Enzyme-linked immunosorbent assay (ELISA) was used to detect the serum interleukin (IL)-6, C-reactive protein (CRP), IL-17, and tumour necrosis factor α (TNF-α) levels. Blood samples of fasting cubital venous (5 mL) were collected within 24 h after admission for all cases. Samples were centrifuged at 2000 g for 15 min, followed with ELISA tested using commercially available kits (IL-6 MBS175877 MyBioSource, IL-17 MBS8123963 MyBioSource, CRP MBS177184 MyBioSource, TNF-α MBS824943 MyBioSource).

Five ml of peripheral elbow vein blood was collected from all subjects at admission, as described earlier.[14,15] Peripheral blood mononuclear cells were isolated. The expression of TIM-3$^+$, PD-1$^+$, CD244$^+$ and FOXP3$^+$ were measured using flow cytometry. After centrifugation (1500 rpm × 5 min), 200 µL of the above cell suspension was added to pre-chilled PBS, washed 2-3 times, and then the cells were resuspended in 100 µL of PBS solution. Further 5 µL of PE-Anti-Human CD4 (MBS2569691, MyBioSource), Anti-CD244 (MBS211252, MyBioSource), Anti--TIM-3 (ab210543, Abcam), Anti-PD-1 (ab52587, Abcam) and Anti-FOXP3 (ab215206, Abcam) were added to the centrifuge tube, and then, incubated for 2 hours at 4°C and centrifuged again (3000 rpm × 10 min). FACS Calibur flow cytometer (BD Biosciences, USA) with Diva software (version 6.1, BD Pharmingen USA) was used to measure the frequency of TIM-3$^+$, PD-1$^+$, CD244$^+$ and FOXP3$^+$ expressing on CD4$^+$ T cells.

Demographic and clinical statistics including age, BMI, gender, smoking, *etc*. were collected. Using an automatic biochemical analyser to a performed whole blood test by Hitachi 7600 of Hitachi Corporation, and lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), ferritin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were recorded.

All data were recorded by median (range) or mean ± SD according to distribution, which was confirmed by Kolmogorov-Smirnov analysis. Using Student's t-test and Mann-Whitney U test to analyse the comparison between the two groups. Chi-square test was used for rates. Pearson's analysis used correlation analysis. ROC curve was used for the analysis of the diagnostic value of circulating T cell subtypes for PM/DM patients with bad prognoses. Logistic regression was performed for risk factors of patients with bad outcomes. A value of $p<0.05$ regarded a significant difference. All data used SPSS 18.0 for statistical analyses.

RESULTS

This study enrolled 82 PM/DM patients and 50 healthy volunteers. Compared to the demographic and clinical data of the two groups, the authors found no significant differences in age, gender, BMI, or smoking proportion between healthy volunteers and PM/DM patients (Table I). The ESR, ALT, AST, LDH, and ferritin concentrations in PM/DM patients were remarkably elevated than that in healthy volunteers ($p<0.05$).

Table I: Clinical characteristics of all participants.

Variable	PM/DM n = 82	Healthy n = 50	p-value
Age, years	50 (33~71)	49 (31~72)	0.266
Gender, female (%)	49 (59.76)	30 (60)	1.000
Gender, male (%)	33 (40.24)	20 (40)	1.000
BMI	24.92 (20.97~29.16)	25.95 (21.25~29.24)	0.171
Smoking, n (%)	33 (40.24)	14 (28)	0.100
ESR (mm/h)	31.99±7.95	8.41±2.72	<0.001
ALT (u/L)	91.08 (65.67~118.33)	16.94 (12.41~20.18)	<0.001
AST (u/L)	93.35±8.67	7.21±2.10	<0.001
LDH (u/L)	434.52±68.19	170.36±27.61	<0.001
Ferritin (ug/L)	195.83±103.40	53.98±12.85	<0.001

P-value comparison between PM/DM patients and healthy volunteers. Mann-Whitney U test and Student's t-test were used for continuous data and the chi-square test was used for rates. Body Mass Index (BMI), lactate dehydrogenase (LDH), erythrocyte sedimentation rate (ESR), aspartate aminotransferase (AST), alanine aminotransferase (ALT).

Then, the authors measured the serum inflammatory factors levels, TIM-3$^+$, CD244$^+$, PD-1$^+$and FOXP3$^+$ expressions in all subjects. The serum levels of TNF-α, IL-6, IL-17, and CRP were remarkably increased in PM/DM patients ($p<0.05$, Figure 1), while the IL-17 and CRP levels in DM patients were markedly enhanced than that in PM patients ($p<0.05$). In addition, the frequency of circulating CD4$^+$ T cells expressing TIM-3$^+$, CD244$^+$, PD-1$^+$ and FOXP3$^+$ were then analysed in different subjects.

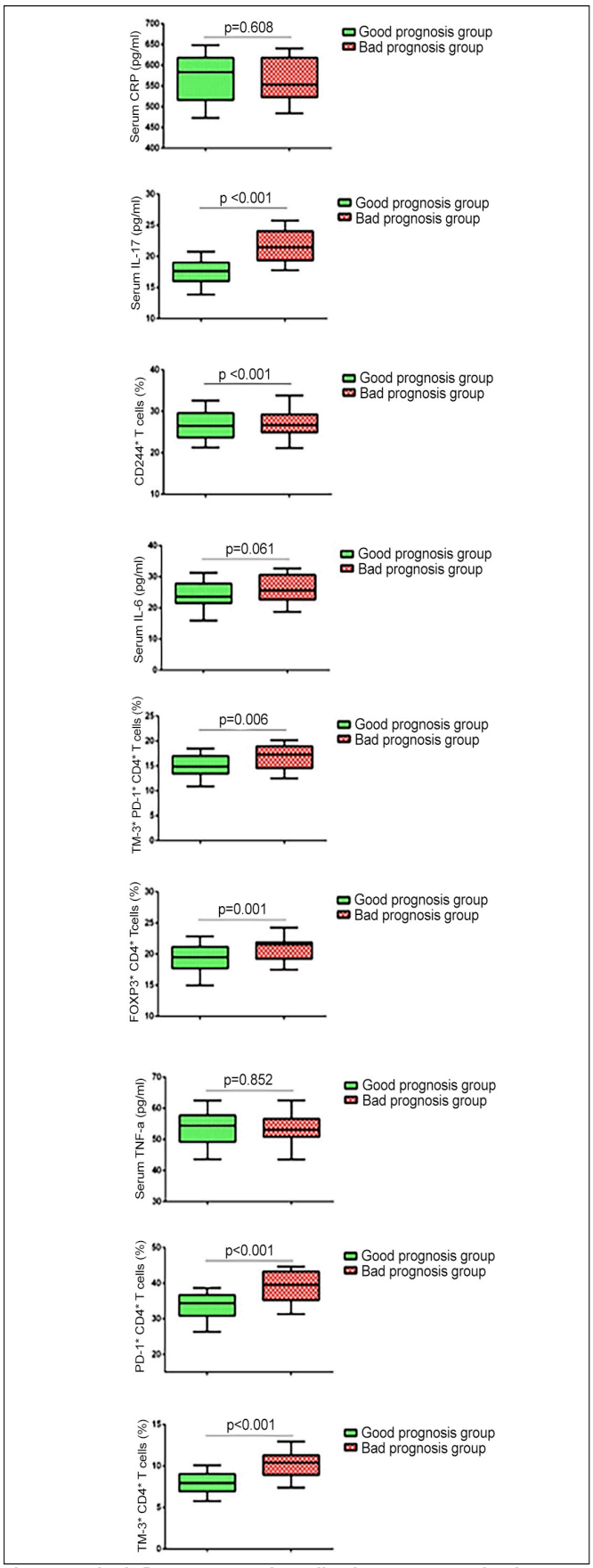

Figure 1: The inflammatory and T cell subtypes expression in PM/DM patients with different prognoses.

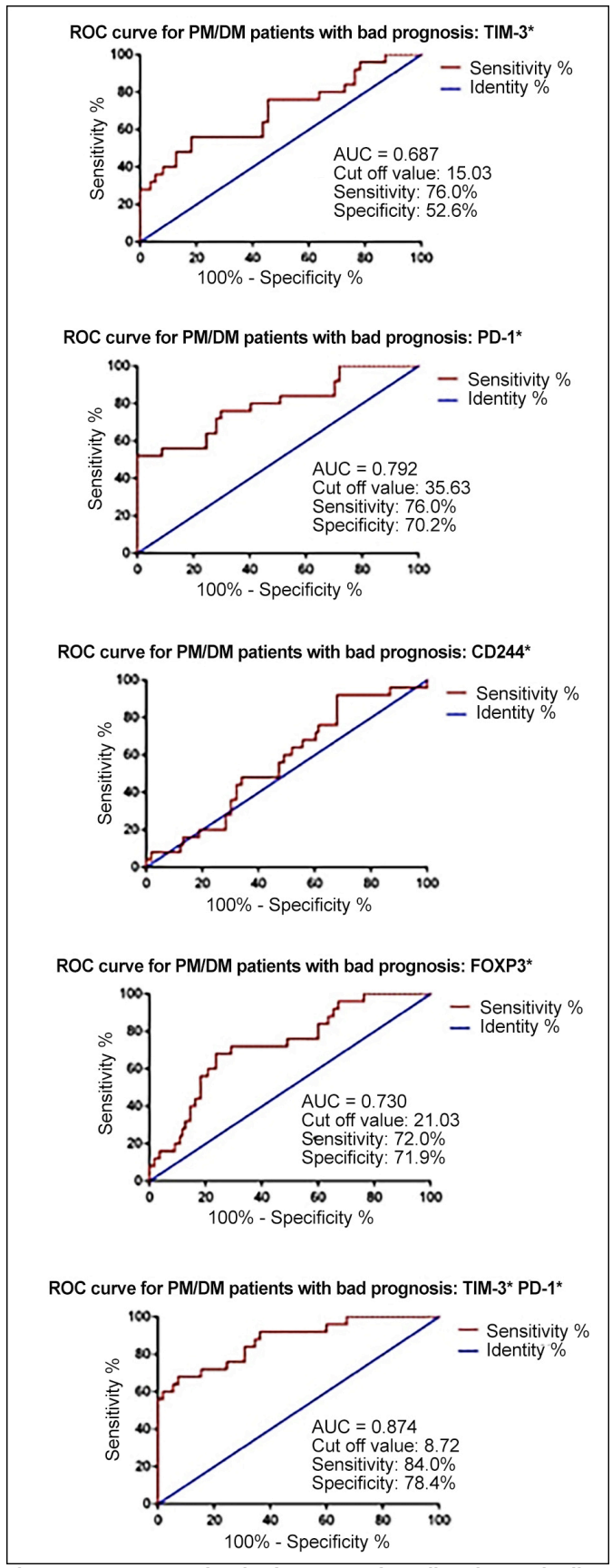

Figure 2: ROC curves for the frequency of T cells subtypes in diagnostic of PM/DM patients with bad prognosis.

Table II: Risk factors of PM/DM patients with bad prognosis by logistic regression analysis.

Variables	Wald	Odds ratio	95% CI	p
Age	1.322	1.038	0.974~1.106	0.250
BMI	2.220	1.217	0.940~1.577	0.136
Smoking	0.017	0.929	0.309~2.794	0.896
ESR	1.587	0.958	0.897~1.0.24	0.208
ALT	0.014	0.998	0.965~1.032	0.904
AST	0.186	1.014	0.953~1.079	0.666
LDH	0.135	0.999	0.991~1.006	0.713
ferritin	3.570	0.995	0.990~1.000	0.059
CRP	0.034	1.001	0.988~1.015	0.854
IL-6	2.739	1.158	0.973~1.378	0.098
TNF-α	0.001	1.003	0.864~1.164	0.971
IL-17	16.391	2.882	1.726~4.810	<0.001
PD-1$^+$	7.751	2.178	1.259~3.767	0.005
TIM-3$^+$	4.469	1.834	1.045~3.217	0.035
CD244$^+$	0.005	1.010	0.754~1.354	0.945
FOXP3$^+$	3.962	2.921	1.017~8.393	0.047
TIM-3$^+$ PD-1$^+$	9.097	15.602	2.617~93.012	0.003

It was observed that the frequencies of TIM-3$^+$, CD244$^+$, FOXP3$^+$, PD-1$^+$ and TIM-3$^+$PD-1$^+$, T cells were all remarkably enhanced in PM/DM patients compared with the healthy control ($p<0.05$, Figure 2). However, no significant difference in the above T cell subtypes between PM and DM patients. Furthermore, Pearson's analysis used correlation analysis. It was found that positive correlation between the IL-17 and the frequency of part of the circulating T cells in CD4$^+$ T cells Table I.

To further investigate the relationship between the frequencies of PD-1$^+$, TIM-3$^+$, T cells and the prognosis of PM/DM patients, the authors divided all patients into a good prognosis group (n=57) and a bad prognosis group (n=25). As shown in Figure 1, the frequencies of PD-1$^+$, TIM-3$^+$ and TIM-3$^+$/PD-1$^+$ T cells were significantly elevated in the bad outcomes group ($p<0.05$). In addition, the serum IL-17 levels and FOXP3$^+$ expression in CD4$^+$ T cells of bad prognosis patients were remarkably higher compared with the good prognosis patients ($p<0.05$). However, no other obvious differences were found.

ROC curves were drawn to assess the diagnostic value of circulating T cell subtypes for PM/DM patients with bad prognoses. The frequency of CD4$^+$ TIM-3$^+$PD-1$^+$ had the best diagnostic value for PM/DM patients with bad outcomes among all T cell subtypes (Figure 2), with an AUC of 0.874, cut-off value of 8.72, the sensitivity of 84.0%, and specificity of 78.4%.

Finally, the risk variables for PM/DM patients with bad prognoses were calculated using binary regression analysis. It was found that IL-17, TIM-3$^+$, PD-1$^+$, FOXP3$^+$and TIM-3$^+$ PD-1$^+$ were the risk factors for PM/DM patients with bad outcomes (Table II).

DISCUSSION

Even though PM and DM can be effectively treated with glucocorticosteroids and immunosuppression, many patients have poor outcomes and even develop serious complications. Therefore, it is important to diagnose PM/DM at an early stage and to accurately assess the prognosis at presentation.[16] In the present research, it was shown that the frequency of circulating CD4$^+$T cells expressing PD-1$^+$ and TIM-3$^+$ could be used to diagnose PM/DM and predict the prognosis of PM and DM patients.

Several biomarkers have been used to assess or early diagnose PM/DM. Previous studies have found abnormal expression of serum DNase1l3[17] levels and muscle tissue vascular endothelial growth factor (VEGF) levels in PM and DM patients,[18] which may be related to clinical features. Zhang *et al.* confirmed that the absolute numbers of Th1, Th2, Th17, CD4$^+$T, and CD8$^+$T decreased significantly, which was significantly correlated with disease activity in PM/DM.[19] Tang *et al.* found that the ratio of CD4$^+$/CD8$^+$ in peripheral blood correlated with post-treatment outcomes, which may be used to predict PM/DM patients' outcomes.[20] Li *et al.* suggested that the frequency of TIGIT$^+$ CD226$^+$ CD4 T cells was significantly enhanced in DM patients and maybe a new therapeutic target.[21] The frequencies of TIM-3$^+$, PD-1$^+$, CD244$^+$, and FOXP3$^+$ T cells were all remarkably enhanced in PM/DM patients compared with the healthy control. The present results showed that the frequencies of TIM-3$^+$, PD-1$^+$, CD244$^+$, and FOXP3$^+$ T cells were all remarkably enhanced in PM/DM patients, and some of our results were consistent with Jayesh *et al.*[22]

Numerous immunological processes, including the control of Th1 and Th17 activity, the induction of tolerance, and the decrease in inflammatory cytokine production, are mediated by TIM-3 and PD-1.[23] According to reports, TIM-3 and PD-1 play a role in the pathogenic development of numerous autoimmune disorders. A clinical study by Luo *et al.* indicated that increasing TIM-3$^+$PD-1$^+$ NK cells was correlated with multiple clinical features in SLE patients.[12] Koohini *et al.*'s clinical study showed that the frequency of Tim-3$^+$ PD-1$^+$ CD4$^+$T cells was obviously elevated in RA patients than that in the controls and positively correlated with the patients' inflammatory factors.[11] The research of Luo and Soliman also verified the above points.[24,25] The present reported findings were similar to these studies, the frequencies of PD-1$^+$, TIM-3$^+$, as well as TIM-3$^+$ PD-1$^+$ T cells were positively correlated with the patient's inflammatory factor IL-6, CRP and PM/DM activity.

This present research also has some limitations. First, it only included a small size of the study population in a single centre study. Secondly, in the T cell subtypes analysis, the authors did not analyse other subtypes, such as CD8$^+$, *etc*. Thirdly, the treatment was not identical for all patients. Finally, more and longer studies are still needed to illustrate the relationship between these T-cell subtypes and the prognosis of PM/DM patients.

CONCLUSION

The frequencies of TIM-3$^+$, PD-1$^+$, CD244$^+$ and FOXP3$^+$ T cells were all remarkably enhanced in PM/DM patients. In addition, the frequency of circulating CD4$^+$T cells expressing TIM-3$^+$PD-1$^+$ could be used to predict the prognosis of PM/DM patients. This study may provide novel research targets for PM/DM treatment.

ETHICAL APPROVAL:

This research has obtained approval from the ethics committee of the Ganzhou people's Hospital.

PATIENTS' CONSENT:

All patients signed informed consent proforma.

COMPETING INTERESTS:
The authors declared that there is no competing interest in this study.

AUTHORS' CONTRIBUTION:
JY, QL, QF, BL, JH, GZ: Substantial contributions to the conception and design of the work, the acquisition and analysis, and interpretation of data for the work.
GZ: Drafting the work and revising it critically for important intellectual content.
All the authors have approved the final version of the manuscript to be published.

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