

PMEL as a Prognostic Biomarker and Negatively Associated With Immune Infiltration in Skin Cutaneous Melanoma (SKCM)

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Summary: Premelanosome protein (*PMEL*) is crucial for the formation of melanosomal fibrils through the transition from stage I to stage II melanosomes. It was used as a target antigen in some adoptive T-cell therapy of melanoma. The correlation of *PMEL* to prognosis and immune cell infiltration level are unknown in melanoma. The *PMEL* expression was evaluated via Tumor Immune Estimation Resource, OncoPrint and Gene Expression Profiling Interactive Analysis (GEPIA). We also evaluate the influence of *PMEL* on overall survival via GEPIA, PrognoScan, and immunohistochemistry in human tissue microarray. The correlation between *PMEL* expression level and immune cell or gene markers of immune infiltration level was explored on Tumor Immune Estimation Resource and GEPIA. *PMEL* expression was significantly higher in skin cutaneous melanoma (SKCM) and SKCM-metastasis in comparison with the other cancers. In SKCM, *PMEL* expression in high levels was associated with poor overall survival. In both SKCM and SKCM-metastasis patients, *PMEL* expression is negatively correlated with the infiltration cells of CD8⁺ T cells, macrophages, and neutrophils. Programmed cell-death protein 1 just showed response rates ranging from 20% to 40% in patients with melanoma, so it is critical to discover a new therapeutic target. *PMEL* is negatively associated with immune cell infiltration and can be as a negative prognosis marker or new immunotherapy target in SKCM and SKCM-metastasis.

Key Words: *PMEL*, immune infiltration, prognosis, SKCM

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Skin cutaneous melanoma (SKCM) incidence is increasing recent years, with an overall rate of 33% for men and 23% women.¹ The programmed cell-death protein 1 (*PD-1*) immune checkpoints of immunotherapy is the mainly treatment measure for advanced-stage melanoma recently, which have dramatically improved patient outcomes, with the median overall survival (OS) of patients increasing from ~9 months to at least 2 years for those with BRAF-V600-mutant disease.² The prognosis of early and advanced melanoma are significantly different, such as the 5-year survival rate for early-stage melanoma can reach 99%,³ but large phase I clinical trials of *PD-1* also showed response rates ranging from 20% to 40% in patients with melanoma.² So when and how to apply immunotherapy is crucial for increasing the OS rate. It is urgent to find out a prognostic marker and new immune infiltration related gene to improve the OS for SKCM patients.

Premelanosome protein (*PMEL*), also recognized as *PMEL17*, glycoprotein 100 (*gp100*), silver homologue, *Me20*, etc. *PMEL*, is a nonmutated “self” antigen expressed by melanocytes, pigmented retinal cells, and most melanoma cells, plays an essential role in the structural organization of premelanosomes. *PMEL* is a melanocyte-specific type I membrane protein mainly expressed in normal skin and eye pigment cells,⁴ and can participate in the formation of melanosomal fibrils through the transition from stage I to stage II melanosomes.⁵ Compared with normal melanocytes, *PMEL* is over-expressed at all stages of melanoma progression,^{6,7} and is a specific marker for melanoma with low-expression in other tissues. Now, *PMEL* has been focused on a target antigen in adoptive T-cell therapy and proven to be its safety and effectiveness.⁸ So, *PMEL*, has represented effective target antigens for the use in adoptive T-cell transfer.⁹ The latest research demonstrates that the *PMEL* domain that forms the amyloid core of the melanoma body is essential for the formation of melanoma,^{10,11} and we can inhibits melanoma cell epithelial to mesenchymal transition, proliferation, migration, invasion, and promotes apoptosis by targeting *PMEL* through down-regulation of the Wnt signaling pathway.^{12,13} These finding suggest the *PMEL* plays a significant role in melanoma formation, invasion, and metastasis of process.

Here, we systematically analyzed *PMEL* expression and correlation with the prognosis of cancers in the databases including OncoPrint, Gene Expression Profiling Interactive Analysis (GEPIA), PrognoScan. Furthermore, we explored the correlation between *PMEL* expression and immune infiltration immune cells including related markers in the tumor microenvironment via Tumor Immune Estimation Resource (TIMER) and GEPIA. The data in this report reveal that the significant correlation between *PMEL* expression and OS of melanoma as well as the degree of immune infiltration.

METHODS

Oncomine Database Analysis

The mRNA expression of *PMEL* in multiple cancers was analyzed via the Oncomine database (www.oncomine.org/resource/main.html).¹⁴ The threshold settings were as follows: gene of *PMEL*, *P*-value of $1E-4$, fold change of 2.0, gene rank of top 10%, and data type of mRNA, respectively.

PrognScan Database Analysis

The relationship between *PMEL* expression and OS in multiple types of cancers was analyzed by the PrognScan database (<http://dna00.bio.kyutech.ac.jp/PrognScan/index.html>), which is a new database for meta-analysis of the prognostic value of genes.¹⁵ The Figure 3E and F with cutoff value 75% and 50%, respectively. The OS curves of cancers significantly related to *PMEL* expression were as follows with *P*-value < 0.05.

GEPIA

The GEPIA database (<http://gepia.cancer-pku.cn>) is an interactive web which can provides key interactive and customizable functions via the analysis of tumors and normal samples based on TCGA and Genotype-Tissue Expression (GTEx) database.¹⁶ And GEPIA was also applied to generate the OS and disease-free survival curves, based on the top-10 over-expressed mRNA levels and log-rank test with group cutoff of median in melanoma.

Using GEPIA to analyze the different expression levels of *PMEL* between the melanoma samples and normal samples in TCGA and GTEx. The threshold settings were as follows: Expression DIY (do it yourself) of boxplot, cancer name of SKCM, match TCGA normal and GTEx data, Jitter size of 0.4, with $l\text{Log}2\text{FC}$ cutoff of 50% and *P*-value cutoff being 1 and 0.01, respectively.

TIMER Database Analysis

TIMER (<https://cistrome.shinyapps.io/timer/>) is a web-accessible resource,¹⁷ which was used to analysis the correlation of *PMEL* expression profiles with the abundance of immune cell infiltration including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells, via gene modules in melanoma. We also further study the interconnections between *PMEL* expression and immune related markers of tumor-infiltrated immune cells through correlation modules in TIMER. All of the immune infiltrated markers have been reported in previous reports.^{18,19} And we also analyzed *PMEL* expression in different types of cancer and compared their different expression between SKCM-tumor and SKCM-metastasis. The left-most panel shows the levels of *PMEL* expression against the degree of tumor purity.²⁰ The levels of *PMEL* expression with 50% cutoff value were demonstrated by \log_2 RSEM.

Immunohistochemistry (IHC) Analysis of Human Tissue Microarray (TMA) and Scoring

The expression of *PMEL* in the human TMA was determined by IHC assay according to the manufacturer's instructions. Primary antibodies were diluted as follows: a rabbit monoclonal (EPR4864) to melanoma *gp100*, and rabbit monoclonal (SP86; Abcam, Hong Kong; 1:100 dilution, cytoplasmic and nuclear staining). The SKCM TMA (Liao Ding Biotechnology Co. Ltd, Shanghai, China) contains 50 cases/100 cores with histopathologic data, including age, sex, tumor location, Clark, Breslow, tumor size, ulcer, nerve invasion, vascular tumor thrombus, TNM grade, and American Joint Committee on Cancer (AJCC) stage.

Briefly, sections 4- μm thick were placed on slides coated with 3-aminopropyltriethoxysilane. Paraf_x0002_fin sections were deparaffinized in xylene and rehydrated through decreasing concentrations of ethanol (100%, 95%, and 85%, 5 min each). Antigens were unmasked by micro_x0002_wave irradiation for 3 min in pH 6.0 citric buffer and cooled at room temperature for 60 minutes. Endogenous peroxidase activity was blocked by incubation of the slides in 3% H_2O_2 /phosphate-buffered saline, and nonspecific binding sites were blocked with goat serum. Melanoma *gp100* staining was observed in the cytoplasm and nucleus of melanoma cells. Staining of melanoma *gp100* was scored according to its extent and intensity (extent \times intensity), and cytoplasm (extent \times intensity)+nucleus (extent \times intensity), similar to the methods described previously.²¹ And high *PMEL* expression (> 0.1875) or low *PMEL* expression (\leq 0.1875) was determined by receiver operating characteristic curve.

Statistical Analysis

Survival curves were depicted using the Kaplan-Meier method and compared using the log-rank test. For calculating the best cutoff points for OS, the X-tile statistical package (version 3.5.0; Yale University, New Haven, CT) was used. X-tile plot illustrates the presence of substantial tumor subpopulations and shows the robustness of the relationship between a biomarker and outcome by construction of a 2-dimensional projection of every possible subpopulation.

RESULTS

The mRNA Expression of *PMEL* in Multiple Cancer Types

To explore the different expression of *PMEL* in tumor and normal samples of multiple cancer types, the TIMER database was used for analysis. *PMEL* expression in bladder urothelial carcinoma, head and neck cancer HPV positive, lung adenocarcinoma, lung squamous cell carcinoma, prostate adenocarcinoma, stomach adenocarcinoma were higher compared to adjacent normal tissues ($P < 0.0001$) (Fig. 1). To better understand the mRNA expression of *PMEL* between tumor and adjacent normal tissues in various human tumors, Oncomine database was adopted. Compared with the normal tissues, higher expression was observed in breast cancer, lung cancer, and melanoma (Fig. 2C). Furthermore, in the TIMER database, we can demonstrate that *PMEL* expression was significantly higher in SKCM and SKCM-metastasis than other cancers, and much higher in SKCM than in SKCM-metastasis ($P < 0.01$) (Fig. 1). To further explore the expression difference of *PMEL* between tumors and normal tissues in melanoma, we analyzed the data in TCGA and GTEx based on the GEPIA database and found that the expression level in SKCM (461 samples) was significantly higher than that in normal tissues (558 samples) with statistics difference ($P < 0.05$) (Figs. 2A, B).

Over-expressed *PMEL* Predicts the Poor OS of SKCM-metastasis Patients

In the GEPIA database, we found that high *PMEL* expression was correlated with poor OS (Fig. 3A). Furthermore, In the Cox analysis of *PMEL* in TIMER database, SKCM-tumor (364 samples, 178 dying) consists of SKCM-primary (95 samples, 25 dying) and SKCM-metastasis patients (269 samples, 153 dying), we found that higher *PMEL* expressions were also dramatically consistent with the poor OS in SKCM and SKCM-metastasis patients ($P < 0.05$) (Figs. 3B, C). Comparing the high and low-expression levels of *PMEL* in various types of cancer with

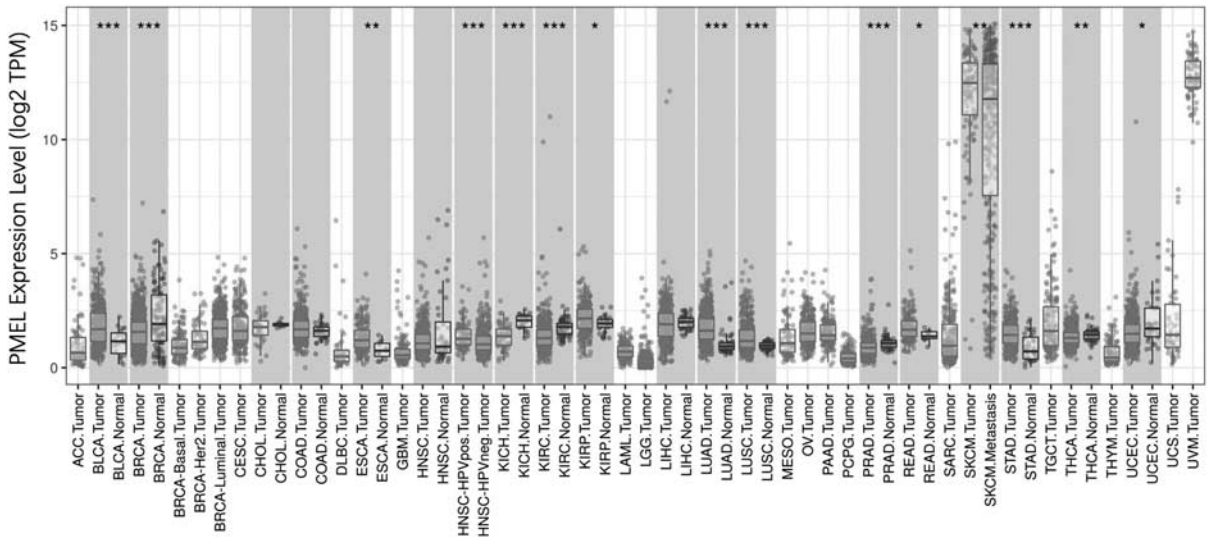


FIGURE 1. *PMEL* expression levels in multiple types of cancers. Compare the expression data of *PMEL* from TCGA using TIMER (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). ACC indicates adenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical and endocervical cancer; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck cancer; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; *PMEL*, premelanosome protein; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; TCGA, The Cancer Genome Atlas; TIMER, Tumor Immune Estimation Resource; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinoma; UVM, uveal melanoma.

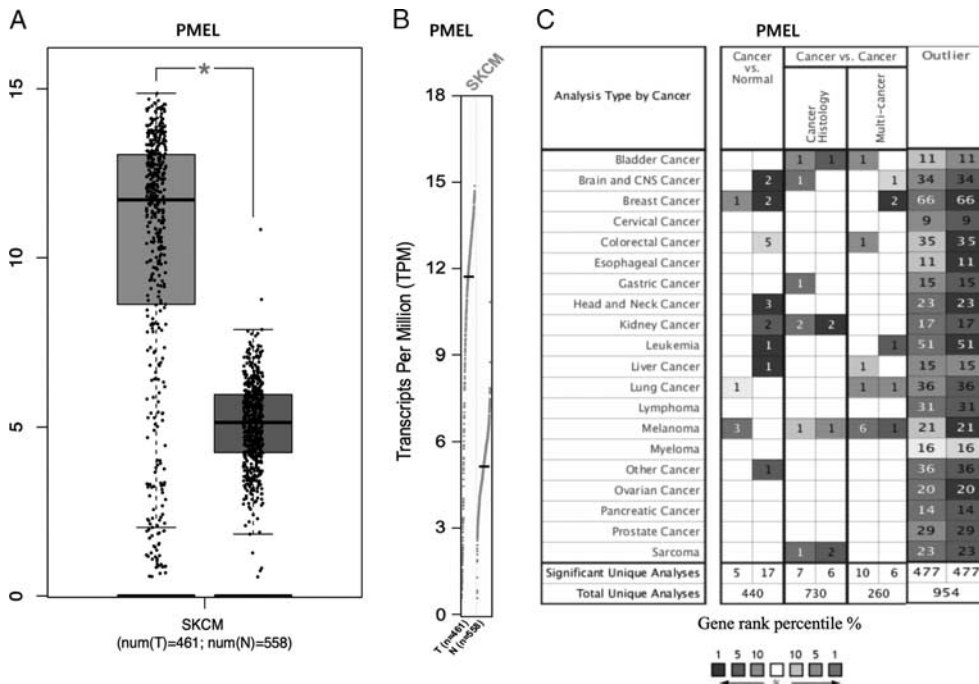


FIGURE 2. A and B, Human *PMEL* expression levels in melanoma from TCGA database were determined by GEPIA. C, The expression levels of *PMEL* in data sets of different cancers between cancer and normal tissues in the OncoPrint database. * $P < 0.01$. CNS indicates central nervous system; GEPIA, Gene Expression Profiling Interactive Analysis; *PMEL*, premelanosome protein; SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas.

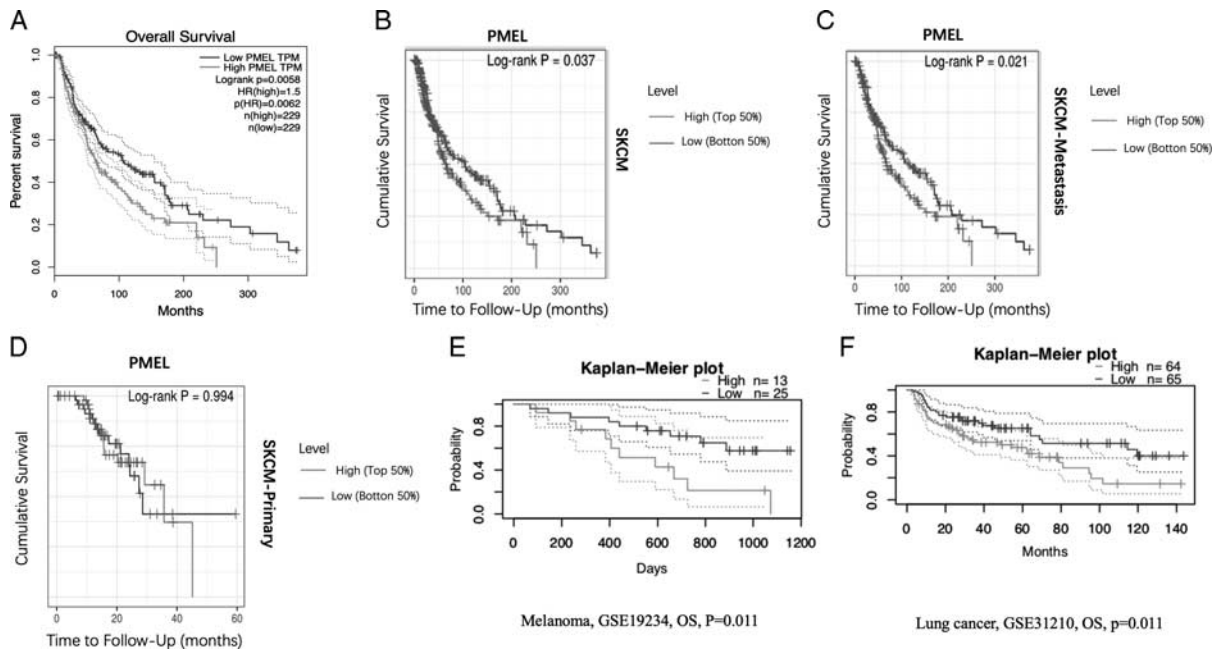


FIGURE 3. The correlation between *PMEL* over-expression gene and OS of SKCM in GEPIA and TIMER database (A–D). A, High *PMEL* expression level corresponded with poor OS prognosis of SKCM in GEPIA database (HR = 1.5, log-rank $P = 0.0058$, cutoff value, 50%). B–D, High *PMEL* expression level correlated with poor OS prognosis of SKCM, SKCM-metastasis and SKCM-primary in TIMER (cutoff value, 50%). The Kaplan-Meier overall survival curves comparing the high and low-expression levels of *PMEL* in various types of cancer in PrognScan (E, F). E and F, High *PMEL* expression level corresponded with poor OS prognosis in melanoma and lung cancer, respectively ($P < 0.05$; cutoff value, 75% and 50%, respectively). GEPIA indicates Gene Expression Profiling Interactive Analysis; HR, hazard ratio; OS, overall survival; *PMEL*, premelanosome protein; SKCM, skin cutaneous melanoma; TCGA, The Cancer Genome Atlas; TIMER, Tumor Immune Estimation Resource.

Kaplan-Meier OS curves in PrognScan, we found that high *PMEL* expression level also corresponded with poor OS prognosis in melanoma and lung cancer ($P < 0.05$) (Figs. 3E, F). So, we could conclude that over-expressed *PMEL* predicts the poor OS of SKCM-metastasis patients. These results lead that *PMEL* can be recognized as a prognostic marker for SKCM patients.

PMEL Expression Levels Correlate With the Clinicopathologic Characteristics of SKCM Patients

To further demonstrate the clinical significance of *PMEL* expression for predicting prognosis in SKCM patients, we tested the expression levels of *PEML* in a human melanoma TMA by IHC, and evaluated the correlation between *PMEL* expression and the clinicopathologic characteristics of the SKCM patients. The expression levels of *PMEL* were graded based on the sum of cytoplasm (extent×intensity) and nucleus (extent×intensity) with score ≥ 0.1875 considered high *PMEL* expression. We can demonstrate that *PMEL* immunoreactivity is mainly in the cytoplasm and nucleus of melanoma cells from the IHC results. Of the 50 TMA cases of SKCM, 45 (90%) of the tissues express detectable *PMEL* expression.

As is shown in the Table 1, following evaluation of the clinical history of the human SKCM specimens, no significant correlations are found between the expression of *PMEL* and patient sex, age, tumor location, Breslow, tumor diameter, nerve invasion or vascular tumor thrombus. In addition, we find that *PMEL* expression is significantly associated with the Clark grade, ulcer and AJCC stage of the SKCM (both

$P < 0.05$) (Table 1). As shown, *PMEL* expression is significantly higher in the higher Clark grade (\geq III) compared with those in the lower Clark grade ($<$ III). Moreover, *PMEL* expression is significantly higher in the higher AJCC stage (III and IV), compared to the lower AJCC stage (I and II).

To analyze the correlation between the *PMEL* expression levels and the clinical prognosis of the SKCM. The patients’ follow-up and clinical outcomes of the SKCM TMA were collected. At the time of last follow-up, among 50 patients in the TMA, 28 had died, the median OS was 50 months (range, 9–59 mo).

Significantly, Kaplan-Meier curves demonstrated that the high *PMEL* expression was significantly associated with a poor OS ($P = 0.016$) (Fig. 4A), and the 3-year OS rate of high and low *PMEL* expression group was about 70.6% and 50%, respectively. Furthermore, *PMEL* expression levels in samples from nonsurvivors are significantly higher than those from survivors (Fig. 4B).

PMEL Expression is Negatively Correlated With Immune Infiltration Cells in SKCM and SKCM-metastasis

It has been confirmed that tumor-infiltrating lymphocyte grade is an independent predictor of survival and sentinel lymph node status in patients with melanoma,^{22,23} which was also demonstrated in other cancers.^{22,24–26} We assessed the correlations of *PMEL* expression with immune infiltration levels between SKCM and SKCM-metastasis from TIMER. It was shown that the *PMEL* expression has significant negative correlations with infiltration cells of CD8⁺ T cell ($r = -0.184$, $P = 1.03e-04$), CD4⁺ T cell ($r = -0.138$, $P = 3.59e$

TABLE 1. The Analysis of Correlation Between the Expression of *PMEL* and Clinicopathologic Characteristics of SKCM Based on TMA

Clinicopathologic Characteristics	Numbers	<i>PMEL</i> Expression [n (%)]		<i>P</i>
		Low	High	
Sex				
Male	31	23 (46.0)	8 (16.0)	0.230*
Female	19	11 (22.0)	8 (16.0)	
Age (y)				
≤60	30	21 (42.0)	9 (18.0)	0.710*
>60	20	13 (26.0)	7 (14.0)	
Tumor location				
Limbs	27	19 (38.0)	8 (16.0)	0.364*
Trunk	5	2 (4.0)	3 (6.0)	
Head and neck	18	13 (26.0)	5 (10.0)	
Clark grade				
I–II	18	16 (32.0)	2 (4.0)	0.026†
III–V	32	18 (36.0)	14 (28.0)	
Breslow (mm)				
≤11	24	17 (34.0)	7 (14.0)	0.680*
>11	26	17 (34.0)	9 (18.0)	
Tumor diameter (cm)				
≤2	28	19 (38.0)	9 (18.0)	0.981*
>2	22	15 (30.0)	7 (14.0)	
Ulcer				
Yes	20	10 (20.0)	10 (20.0)	0.026*
No	30	24 (48.0)	6 (12.0)	
Nerve invasion				
Yes	20	14 (28.0)	6 (12.0)	0.804*
No	30	20 (40.0)	10 (20.0)	
Vascular tumor thrombus				
Yes	26	17 (34.0)	9 (18.0)	0.680*
No	24	17 (34.0)	7 (14.0)	
AJCC stage (TNM)				
I–II	29	23 (46.0)	6 (12.0)	0.044*
III–IV	21	11 (22.0)	10 (20.0)	

Bold values indicate statistical significance ($P < 0.05$).

*Pearson χ^2 test.

†Fisher exact test.

AJCC indicates American Joint Committee on Cancer; *PMEL*, pre-melanosome protein; SKCM, skin cutaneous melanoma; TMA, tissue microarray.

–03), macrophage ($r = -0.307$, $P = 2.47e-11$), neutrophil ($r = -0.349$, $P = 2.22e-14$) and dendritic cell ($r = -0.129$, $P = 6.56e-03$) in SKCM (Fig. 5A), and CD8⁺ T cell ($r = -0.157$, $P = 3.95e-03$), macrophage ($r = -0.279$, $P = 1.09e-07$) and neutrophil ($r = -0.315$, $P = 1.59e-09$) in SKCM-metastasis (Fig. 5B). So the result shows that the *PMEL* expression level have a significant negative correlation with the immune infiltration level, especially in the CD8⁺ T cell, macrophage, and neutrophil. These findings strongly suggest that *PMEL* affects patient survival via interacting with immune infiltration in SKCM and SKCM-metastasis.

Furthermore, we further demonstrated that the correlation between immune infiltration and survival rate of SKCM and SKCM-metastasis patients in TIMER database. After the adjustment of clinical factors (age, sex, race, stage, tumor purity), we concluded that lower infiltrations of B cell, CD8⁺ T cell, neutrophil, and dendritic cell were significantly associated with a poor cumulative survival rate in both SKCM and SKCM-metastasis patients ($P < 0.05$) (Figs. 6A, B). So, how can we do to promote the immune infiltration via influencing the *PMEL*?

The Analysis of Correlation Between *PMEL* Expression and Immune Markers

To further illuminate the correlation between *PMEL* expression and immune infiltration cells, we study the relation between the *PMEL* expression and immune markers of relative immune infiltration cells of SKCM and SKCM-metastasis in TIMER and GEPIA database. The relative immune infiltration cells are mainly including CD8⁺ T cell, monocyte, tumor-associated macrophage (TAM), M1 macrophage, M2 macrophage, neutrophils, dendritic cell, Th1, Th2, Tfh, Th17, Treg, exhausted T cell (Table 2). After correlation analysis adjusted by purity, we demonstrated that the expression of *PMEL* is significantly negative correlated with the amount of immune infiltration relative immune markers with 36 of 48 immune cell markers in SKCM and 25 of 48 immune cell markers in SKCM-metastasis ($P < 0.05$) (Table 2). The difference of correlation between the *PMEL* expression and immune markers of relative immune infiltration cells demonstrates that *PMEL* expression may play a negative role in the SKCM.

We can see a negative correlation between *PMEL* expression and monocyte, macrophage and TAM of SKCM and SKCM-metastasis in TIMER database (Table 2). And we also find a low immune infiltration level of macrophage was associated with high *PMEL* expression level (Figs. 4B, 5A). But we did not find macrophage associated with a poor survival rate of SKCM, SKCM-primary and SKCM-metastasis patients. So, those results can tell us that *PMEL* influences more function of macrophage than quantity or *PMEL* on macrophage infiltration might be coordinated by some signals which have less effect on the OS.

Moreover, high *PMEL* expression in SKCM also relates to low infiltration level of CD8⁺ T cell (*CD8A*, *CD8B*), neutrophils (*CD11b*), dendritic cell (*HLA-DPB1*, *HLA-DQB1*, *HLA-DRA*, *HLA-DPA1*, *BDCA-1*, *BDCA-4*), Th1 (*T-bet*, *STAT4*, *IFN- γ*), Th2 (*GATA3*, *STAT6*, *STAT5A*, *IL13*), Tfh (*BCL6*, *IL21*), Th17 (*STAT3*), Treg (*CCR8*, *STAT5B*, *TGF β*) and exhausted T-cell markers (*LAG3*, *TIM-3*, *GZMB*), and the *PD-1* is the only 1 positive immune marker correlated to *PMEL* in SKCM and SKCM-metastasis ($P < 0.05$) (Table 2). In the SKCM-metastasis, compared with the SKCM, the correlation between the *PMEL* and immune marker including CD8⁺ T cell (*CD8A*, *CD8B*), dendritic cell (*HLA-DPB1*, *HLA-DQB1*, *HLA-DPA1*), Th1 (*T-bet*, *IFN- γ*), Tfh (*IL21*), Treg (*STAT5B*), and exhausted T-cell markers (*LAG3*, *GZMB*) is not confirmed again ($P > 0.05$) (Table 2). Compared with the difference, we guess that *PMEL* may influence the metastasis of SKCM via interacting the partial function of CD8⁺ T cell, dendritic cell, Th1, Tfh, Treg, and exhausted T-cell markers.

DISCUSSION

As a melanocyte-specific type I membrane protein, *PMEL*, has been considered as a melanocyte biomarker in the diagnosis of primary cutaneous melanoma and in the identification of melanoma metastasis biopsy to sentinel lymph node.^{27,28} Before the *PMEL* protein entry into stage I melanosomes, some of fraction of the protein is transiently presented to the cell surface.^{29–31} It is interesting to note that, the process of *PMEL* transiently migrating to cell surface makes it possible for immune cells and antibody drug conjugate to target melanoma cell.³² The highly restricted expression of *PMEL* in normal tissues makes it become a potential drug target for melanoma. Immunology including the cancer vaccine, immune checkpoints

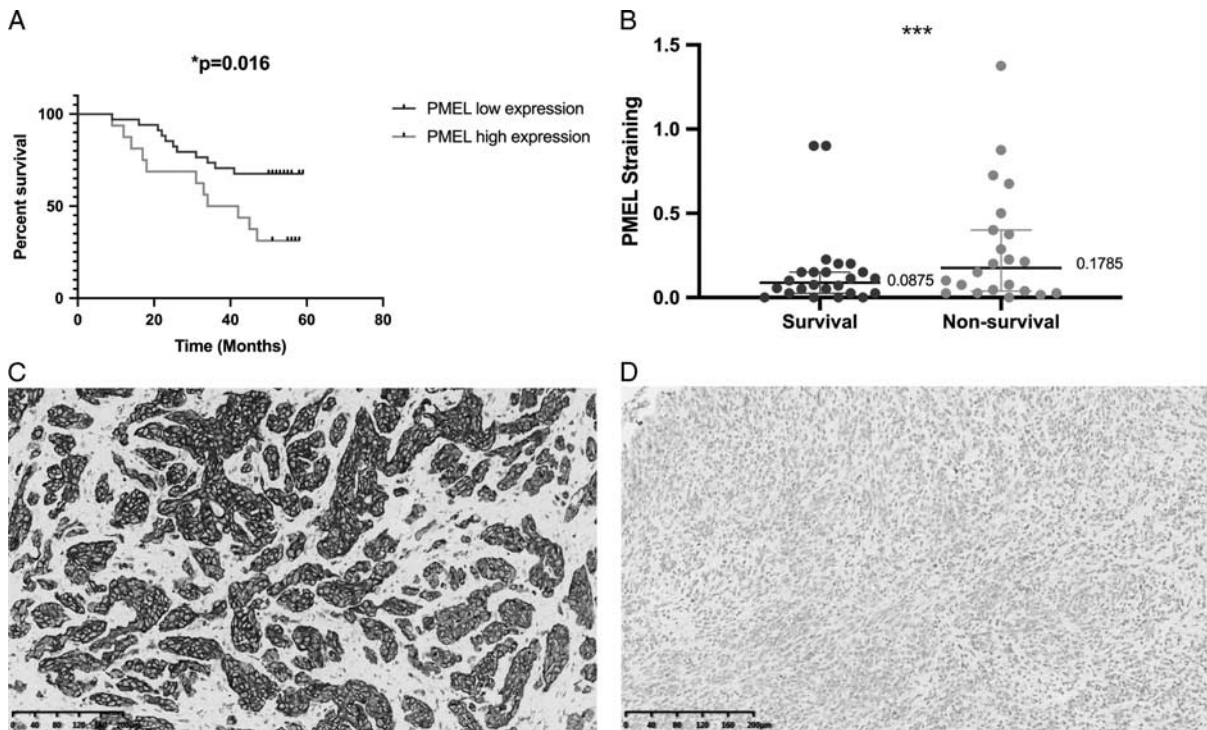


FIGURE 4. *PMEL* expression levels associate with clinicopathologic characteristics of SKCM patients. *PMEL* levels in SKCM tissue microarray were evaluated by immunohistochemistry, and the relationship between *PMEL* expressions and overall survival was analyzed. A, Kaplan-Meier survival curve of SKCM patients with high *PMEL* expression (> 0.1875) or low *PMEL* expression (\leq 0.1875). B, Distribution of *PMEL* staining scores in the SKCM tissue samples from survival and nonsurvival patients. *** $P < 0.001$. C and D, Representative images of high and low *PMEL* expression level based on different immunohistochemical staining intensities. *PMEL* indicates premelanosome protein; SKCM, skin cutaneous melanoma.

inhibitor is a young and growing field, and melanoma was recognized as a significant cancer model due to its obvious immunogenicity. *PMEL*, also known as *gp100*, has been used as a peptide vaccine of antimelanoma for several decades due to the ability to activate the CD8⁺ T-cell rapidly.^{33,34} So, we can guess that a significant positive correlation between *PMEL* expression and immune infiltration cells can be demonstrated. But is this theoretical guess right?

Here, in our present research, we found that the expression of *PMEL* was negatively associated with the immune infiltration cells such as CD8⁺ T cell, macrophage, neutrophil, and dendritic cell in the SKCM and SKCM-metastasis. In addition, high expression of *PMEL* was associated with a poorer OS compared with the lower expression patients in SKCM, which indicated that the *PMEL* can be recognized as a predictor of poor prognosis. Furthermore, our study showed that immune infiltration levels and various immune infiltration related gene markers are mostly negative correlated with the *PMEL* expression level. Thus, our study provided a chance to clarify the inner correlation between *PMEL* expression and immunomelanoma, and the value as a melanoma prognostic biomarker.

Immune infiltration cells of tumor microenvironment are significant determinant for immune response and outcomes in various cancers including melanoma.^{35,36} Here, we also demonstrated that higher immune infiltration level such as B cell, CD8⁺ T cell, neutrophil, and dendritic cell were associated with a better OS in the SKCM and SKCM-metastasis patients (Fig. 6), which is consistent with the

previous research. But in our study, higher *PMEL* expression was associated with lower immune infiltration level including CD8⁺ T cell, macrophage, neutrophil and dendritic cell in the TIMER database (Fig. 5). It was also found that most of the immune infiltration gene marker have a moderate or strong negative correlation with the *PMEL* expression (Table 2), which implicate the negative role of *PMEL* in regulating tumor immunology in SKCM. First, gene markers of CD8⁺ T cell including CD8A and CD8B demonstrated a negative correlation with *PMEL* expression level, which may explain the phenomenon of poorer prognosis correlated with higher *PMEL* expression. Interesting, the metastasis of melanoma was most occurred in the lymph nodes, which pooling large numbers of lymphocytes. These may provide an explanation for the negative correlation disappearing in the SKCM-metastasis patients. Second, gene markers of M1 macrophage such as NOS2, IRF5, and PTGS2 showed a weak correlation with *PMEL* expression but a strong correlation exhibited for the M2 macrophage including CD163, VSIG4, MS4A4A (Table 2), which indicated the potential regulatory function of *PMEL* in the polarization of TAM. In addition, a moderate to strong negative correlations between *PMEL* expression level and infiltration level of DCs can be demonstrated in this study, which may be the reason why we should increase cross-presentation of immature dendritic cells to *gp100* specific CD4 T cells via modifying glycosylation on *gp100*.³⁷ However, our study also indicated that *PMEL* play a significant negative role in activating Tregs and inducing T-cell exhaustion. The higher *PMEL* expression negatively

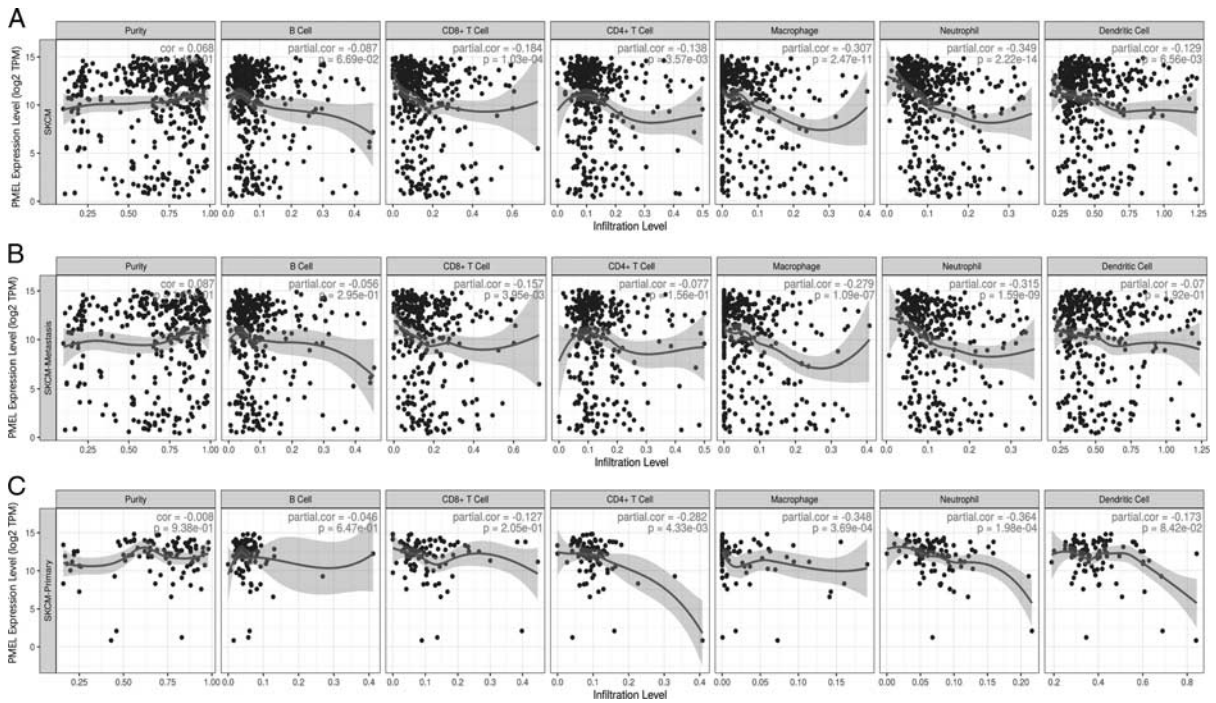


FIGURE 5. The analysis of correlation between *PMEL* expression level and immune infiltrates in SKCM (A), SKCM-metastasis (B) and SKCM-primary (C). A, The negative correlations between *PMEL* expression and immune infiltration of CD8⁺ T cell, CD4⁺ T cell, macrophage, neutrophil, and dendritic cell in SKCM. B, The negative correlations between *PMEL* expression and immune infiltration of CD8⁺ T cell, macrophage, and neutrophil in SKCM-metastasis. C, The negative correlations between *PMEL/SILV* expression and immune infiltration of CD4⁺ T cell, macrophage, and neutrophil in SKCM-primary. *PMEL* indicates premelanosome protein; *SILV*, silver homologue; SKCM, skin cutaneous melanoma.

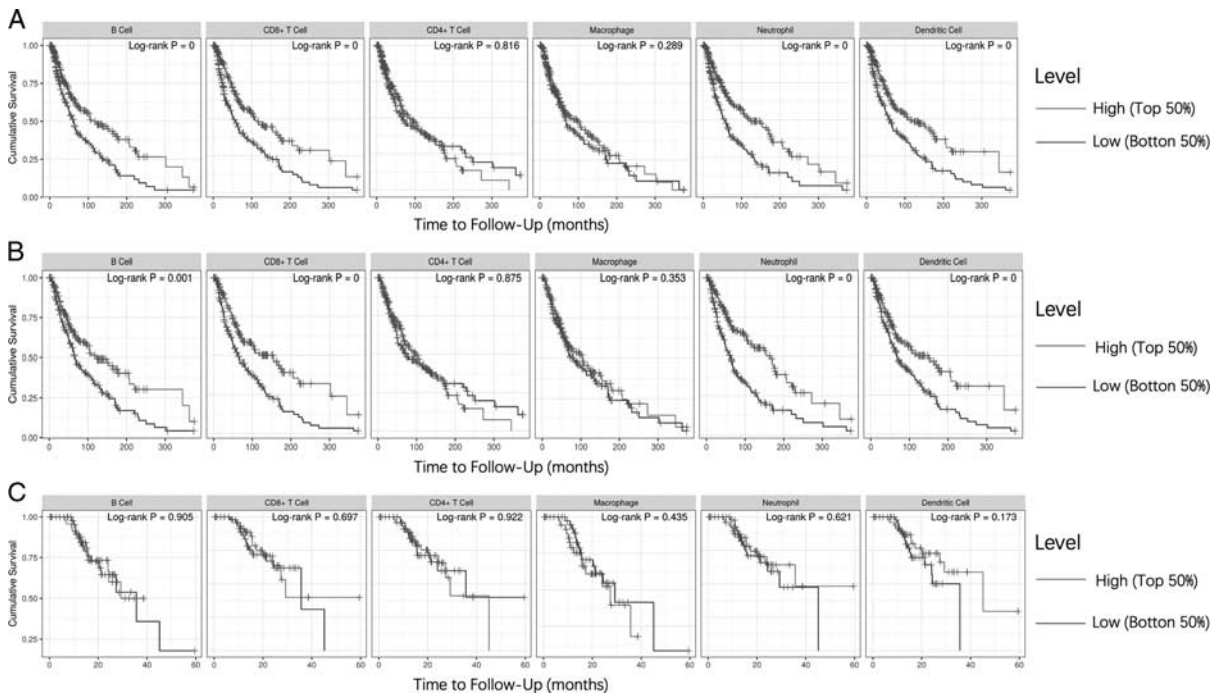


FIGURE 6. The correlation between immune infiltrates and overall survival rate of SKCM (A), SKCM-metastasis (B) and SKCM-primary (C) patients in TIMER. The indicates were divided into high and low groups with cutoff 50%. SKCM indicates skin cutaneous melanoma; TIMER, Tumor Immune Estimation Resource.

TABLE 2. Correlation Analysis Between *PMEL/SILV* and Relate Gene Markers of Immune Cells in TIMER Database

Description	Gene Markers	SKCM		SKCM-metastasis	
		Correlation	P	Correlation	P
CD8 ⁺ T cell	CD8A	-0.139	**	-0.106	0.0412
	CD8B	-0.116	0.012	-0.078	0.134
Monocyte	CD86	-0.283	***	-0.249	***
	CD115 (CSF1R)	-0.335	***	-0.307	***
TAM	CD14	-0.302	***	-0.272	***
	CCL2	-0.305	***	-0.287	***
	CD68	0.085	0.072	0.088	0.093
M1 macrophage	IL10	-0.238	***	-0.205	***
	NOS2	0.045	0.328	0.075	0.154
	IRF5	-0.076	0.102	-0.033	0.526
M2 macrophage	PTGS2	-0.281	***	-0.308	***
	CD163	-0.297	***	-0.269	***
	VSIG4	-0.277	***	-0.246	***
Neutrophils	MS4A4A	-0.297	***	-0.267	***
	CEACAM8	-0.075	0.105	-0.09	0.084
	CD11b (ITGAM)	-0.244	***	-0.208	***
Dendritic cell	CCR7	-0.035	0.443	-0.033	0.534
	HLA-DPB1	-0.172	**	-0.139	*
	HLA-DQB1	-0.126	*	-0.092	0.078
	HLA-DRA	-0.21	***	-0.177	**
	HLA-DPA1	-0.143	*	-0.099	0.057
	BDCA-1 (CD1C)	-0.219	***	-0.205	***
	BDCA-4 (NRP-1)	-0.522	***	-0.539	***
Th1	CD11C (ITGAX)	-0.075	0.104	-0.024	0.639
	T-bet (TBX21)	-0.144	*	-0.117	0.025
	STAT4	-0.34	***	-0.298	***
	STAT1	-0.105	0.023	-0.065	0.215
	IFN- γ (IFNG)	-0.135	*	-0.11	0.035
	TNF- α (TNF)	-0.107	0.020	-0.115	0.027
	GATA3	-0.194	***	-0.212	***
Th2	STAT6	0.192	***	0.237	***
	STAT5A	0.243	***	0.305	***
	IL13	-0.174	**	-0.184	**
Tfh	BCL6	-0.39	***	-0.365	***
	IL21	-0.133	*	-0.1	0.055
Th17	STAT3	-0.16	**	-0.146	*
Treg	IL17A	0.078	0.091	0.036	0.497
	FOXP3	-0.101	0.028	-0.088	0.094
	CCR8	-0.219	***	-0.186	**
T cell exhaustion	STAT5B	-0.091	0.048	-0.053	0.311
	TGF β (TGFB1)	-0.303	***	-0.31	***
	<i>PD-1</i> (SNCA)	0.539	***	0.582	***
	CTLA4	-0.061	0.184	-0.079	0.128
	LAG3	-0.13	*	0.098	0.060
	TIM-3 (HAVCR2)	-0.226	***	-0.191	**
	GZMB	-0.118	0.010	-0.111	0.038

None indicates without adjustment; Purity, adjusted by tumor purity; *PMEL*, premelanosome protein; *SILV*, silver homologue; SKCM, skin cutaneous melanoma; SKCM-metastasis, the metastasis of skin cutaneous melanoma; TAMs, tumor-associated macrophages; Tfh, follicular helper T cell; Th1, T helper cell 1; Th2, T helper cell 2; Treg, regulatory T cell; TIMER, Tumor Immune Estimation Resource.

* $P < 0.01$.

** $P < 0.001$.

*** $P < 0.0001$.

correlates with the expression of Tregs and exhausted cells gene markers such as CCR8, STAT5B, TGF β , LAG3, TIM-3, and GZMB (Table 2). The possible hypothesis or explanation about *PMEL* expression negative correlation of immune cell infiltration is that as a normal, nonmutated “self”-protein, *PMEL*, presents poor immunogenicity and apparent hyporesponsiveness to the human immune,^{38,39} so autologous *PMEL* is challenging to stimulate effective immune cell infiltration. Poor immunogenicity of *PMEL* antigens is due to the instability of the peptide-major

histocompatibility complex,³⁹ and vaccination with autologous *PMEL* will not trigger these CD8⁺ T cells unless the vaccine is able to significantly raise the amount of peptide-major histocompatibility complex class I complex on professional antigen presenting cells to a level high enough to surpass the TCR threshold.³⁸

Thus, a positive expression correlation between *PMEL* and *PD-1* was confirmed in our study, and the high expression of *PMEL* is also associated with the increasing *PD-1* expression, which explain the better response for *PD-1* in melanoma.

Based-*PMEL* tumor vaccine is one of the important explorations of new treatment methods for melanoma. Studies have found that this type of vaccine can play a better synergistic effect with immune checkpoint inhibitors (*PD-1*, etc.).^{40,41} Finally, significant correlations can be found between *PMEL* expression and several markers of T helper cells including Th1, Th2, Tfh, Th17 in SKCM. These correlations could give us a potential mechanism to explain if and how *PMEL* regulates the T cells function in SKCM. In conclusion, all these finding indicated that *PMEL* play a significant role in regulating the immune infiltration function in SKCM.

Melanoma vaccination with various forms combined with *PMEL* could educate immune system to recognize and eradicate melanoma cells,^{42,43} in particular to activate *PMEL*-specific CD8⁺ T cells against *PMEL* peptides. *PMEL*-based melanoma vaccines have been tested extensively in clinical trials.^{44–48} So high *PMEL* expression can more efficiently and strongly react with CD8⁺ T cells stimulated by *PMEL*-based vaccine, and the effect of anti-melanoma immune response will be better.

Actually, the *PMEL* is not only highly expressed in melanoma, but also found in other tumor tissues such as glioblastoma multiforme primary cell line,⁴⁹ breast cancer.⁵⁰ In our study, we examined the expression level of *PMEL* mRNA and its correlation with the prognosis of different cancers in Oncomine, TIMER and GEPIA databases. Compared with the normal tissues or the other cancers, the *PMEL* expression level was highly expressed in melanoma including SKCM on the Oncomine database. From the results of the PrognScan database, the high *PMEL* expression level can be used as an independent potential factor for poor prognosis in melanoma and lung cancer ($P < 0.05$) (Figs. 3E, F). The unique high expression level of *PMEL* in melanoma and the obvious negatively correlation between *PMEL* mRNA expression level and prognosis indicate that *PMEL* will be a potentially better prognostic biomarker in melanoma.

Recent studies demonstrated that the critical *PMEL* domain has a significant effect on the formation of melanoma, and high *PMEL* expression plays a positive role in melanoma invasion and metastasis of process.^{10,12,13} In summary, higher *PMEL* expression level correlates with poorer prognosis and lower immune cell infiltration level in CD8⁺ T cell, macrophage, neutrophil, and dendritic cell in the SKCM and SKCM-metastasis. Thus, the correlation between *PMEL* expression level and immune infiltration markers in SKCM, may provide an explanation that *PMEL* influence the prognosis of SKCM through inhibiting the infiltration level of macrophage, TAMs, DCs, Tregs, exhausted T cells, and so on. Therefore, *PMEL* is associated with immune cell infiltration and can be as a prognosis marker or new immunotherapy target in SKCM and SKCM-metastasis.

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Conflicts of Interest/Financial Disclosures

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