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Epstein-Barr Virus-induced Gene 3 as a Novel Biomarker in Metastatic Melanoma With Infiltrating CD8$^+$ T Cells: A Study Based on The Cancer Genome Atlas (TCGA)

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Abstract. *Background/Aim: Epstein-Barr virus-induced gene 3 (EBI3) is an immunomodulatory protein-coding gene. So far, the prognostic role of EBI3 in human metastatic melanoma has been unclear. This study aimed to evaluate the EBI3 expression as a potential biomarker using the public database with tumor-infiltrating lymphocytes (TILs) data. Materials and Methods: Survival analyses were performed in the database of The Cancer Genome Atlas (TCGA) and GSE65904, GSE19234, GSE22153, and GSE22154. The mRNA levels, the distribution pattern of TILs, and the estimated fractions of TILs from the TCGA database were integrated. Results: Higher EBI3 expression in tumors was significantly associated with longer overall survival in TCGA and the other independent cohorts. Interestingly, the patients with high EBI3 expression had a brisk pattern of TILs and increased CD8$^+$ T cells over regulatory T cells with less pigmentation-related gene expressions. Conclusion: EBI3 could serve as a novel biomarker in metastatic melanoma with a favorable TILs profile.*

Cutaneous melanoma is an aggressive malignancy of melanocytes. Melanoma is the third most common cutaneous malignant tumor, accounting for less than 5% of cases (1). However, the majority of skin cancer-associated mortality is caused by melanoma (2). The tumor immune microenvironment (TIME) of melanoma is enriched with tumor-infiltrating lymphocytes (TILs) (3). TILs have a clinical role as a prognostic predictor in melanoma (4). Investigation of the immune context of the TIME is important for understanding its impact on response to therapy (5).

Epstein-Barr virus-induced gene 3 (EBI3) is a member of the interleukin-12 (IL-12) family and forms a heterodimer either with the IL-27p28 subunit to form IL-27 or with IL-12p35 subunit to form IL-35 (6). Both IL-27 and IL-35 can affect the TIME by modulating regulatory T cells (Treg) and cytotoxic T cells (7-10). The prognosis of multiple cancers, including breast cancer (11), colorectal cancer (6), lung cancer (12), and cervical cancer (13) is associated with EBI3 expression in tumors. However, the prognostic role of EBI3 in melanoma has not been investigated so far.

The current study aimed to provide evidence on the role of *EBI3* expression as a novel biomarker in metastatic melanoma by characterizing the TIME using the publicly available database.

Materials and Methods

Survival analysis with EBI3 mRNA level. Clinicopathological data was obtained from The Cancer Genome Atlas (TCGA) database in the cBioportal (https://www.cbioportal.org/) (14, 15). Survival analysis with gene expression was performed using the dataset of TCGA and GSE65904 (16). The patients with upper 50% expression of *EBI3* were defined as *EBI3* high, and the others were *EBI3* low, in each dataset. For visualizing the survival data, the Kaplan–Meier plotting and log-rank test was used to compare the survival distribution between groups. The *EBI3* gene expression as a continuous variable was included for the multivariate Cox regression analysis with the other parameters such as age, sex (male *vs.* female), and disease stage at initial diagnosis on TIMER (https://cistrome.shinyapps.io/timer/) (17). Meta survival analysis of *EBI3* as a prognostic marker on the overall survival was conducted on GENT2 (http://gent2.appex.kr/gent2/) (18). The included database in the meta-survival analysis were GSE19234 (19), GSE22153, and GSE22154 (20).

Spatial distribution of TILs. The data of spatial distribution patterns of TILs and computed indices of TILs clustering patterns (Ball and Hall, Banfield and Raftery, C, and determinant ratio) used in Saltz *et al.* (21) were downloaded from the Genomic Data Commons (GDC) Data Portal (https://gdc.cancer.gov/about-data/publications/tilmap). Samples of 'Indeterminate' pattern were excluded. The distribution pattern of TILs between *EBI3* high and low patients was compared by Fisher's exact test.

Estimated fractions of TILs. The estimated TILs fraction by the quanTIseq method (22) was downloaded from TIMER2.0 (http://timer.comp-genomics.org/) (23).

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Pigmentation-related gene signature. The following gene set was used as pigmentation-related gene signature (24): *GPR143*, *SLC45A2*, *OCA2*, *MC1R*, *PMEL*, *RAB27A*, *MLANA*, *TYRP1*, *DCT*, *TYR*, *SNCA*, *MITF*, *GPNMB*, *TRPM1*, *MCOLN3*, *RAB38*, *MITF*. Spearman correlation analysis was conducted on Gene Expression Profiling Interactive Analysis 2 (GEPIA2, http://gepia2.cancer-pku.cn/) (25).

Statistical analysis. Spearman's rho was used for correlation analyses. For graphs and statistical analyses (Mann-Whitney test, Fisher's exact test, chi-square test, and principal component analysis) were used. Analyses were performed using the R freeware (http://www.r-project.org) and GraphPad Prism (v. 9.2.0) software (GraphPad Software Inc., CA, USA). All *p*-values were two-sided, and a *p*-value ≤0.05 was considered statistically significant.

Results

Prognostic EBI3 gene expression in metastatic melanoma. Clinicopathologic characteristics of the patients were shown in Table I. The parameters except for sex (p=0.015) and pT stage (p=0.0001) showed no significant difference (Table I). The survival analysis with *EBI3* gene expression data was performed in the metastatic melanoma dataset from TCGA. The *EBI3*-high patients had longer overall survival (OS) [hazard ratio (HR)=0.586, 95% confidence interval (CI)=0.438-0.783, p=0.0004, Figure 1A]. A similar prognostic role was found in another independent cohort (GSE65904), revealing that the *EBI3* gene expression was a favorable factor on disease-specific survival (DSS) (HR=0.589, 95% CI=0.398-0.871, p=0.0068, Figure 1B). Moreover, the meta-survival analysis of *EBI3* on GENT2 (18) revealed that the higher *EBI3* expression correlated with better OS in both fixed and random effect model (HR=0.84, 95% CI=0.72-0.97, HR=0.82, 95% CI=0.68-0.99, respectively, Figure 2). In TCGA dataset, the multivariate Cox regression analysis also showed that *EBI3* gene expression was an independent favorable prognostic factor (HR=0.737, 95% CI=0.652–0.834, p<0.0001, Table II).

Spatial pattern of tumor-infiltrating lymphocytes by EBI3 gene expression. *EBI3*-encoded protein is immunomodulatory by forming heterodimers relating to IL-27 and IL-35 (26). Therefore, the current study investigated the profile of TILs between *EBI3* high *vs*. low groups. First, the spatial patterns of TILs (21) were investigated; in the *EBI3* high group, about 73% (108/148) of the patients had a brisk distribution pattern (*i.e*., brisk multifocal or brisk focal). Contrarily, in the *EBI3* low group, only 47% (69/146) of the included patients had a brisk pattern. The distribution pattern of TILs between *EBI3* high and low patients was significantly different (p=9.203e-06, Fisher's exact test, Figure 3A).

Spatial patterns of TIL clustering can be described by several indices such as Ball and Hall, Banfield and Raftery, C, and determinant ratio (21). In the melanoma dataset of TCGA, better prognosis correlated with a higher Banfield and Raftery index (21). In the current study, the adjusted Banfield-Raftery and adjusted determinant ratio indices were significantly higher in the *EBI3* high patients (Figure 3B-C), suggesting that *EBI3* high patients had favorable TIL clusters in the tumor tissue. In contrast, the other indices (adjusted Ball and Hall and adjusted C) were not significantly different (data not shown).

Antitumor TILs profile and activity with EBI3 gene expression. *EBI3* supports antitumor immunity with dominant $CD8^+$ T cells over regulatory T cells (Treg) *in vivo* (26). Therefore, it was hypothesized that *EBI3* expression in human melanoma tissue is also associated with TILs. Fractions of TILs were estimated by the quanTIseq method (22). In TCGA dataset, *EBI3* high patients had a profound shift of TIL populations compared to *EBI3* low patients as shown by the principal component analysis (Figure 4A). The *EBI3* mRNA level positively correlated with the fractions of B cells, $CD8^+$ T cells, Treg, M1/M2 macrophages, and $CD8^+$

Table I. *Clinicopathological characteristics between Epstein-Barr virus-induced gene 3 (EBI3) high and low patients on TCGA dataset*.

Variables	*EBI3* low	*EBI3* high	*p*-Value*
No. of patients (%)	182 (50%)	181 (50%)	
Age**, years	56.5 (15-87)	57 (18-86)	0.953
Gender			0.015
Male	126	102	
Female	56	79	
Race			0.549
White	175	174	
Other (Asian or African American)	2	4	
NA	5	3	
TNM stage			0.112
Stage 0	4	3	
Stage I	37	38	
Stage I/II (NOS)	6	7	
Stage II	47	25	
Stage III	65	76	
Stage IV	8	13	
NA	15	19	
pT stage			0.0001
T0/Tis	8	23	
T1	12	29	
T2	44	29	
T3	42	37	
T4	43	22	
NA/Tx	33	41	

TCGA, The Cancer Genome Atlas; NOS, not otherwise specified; Tis, melanoma *in situ*; NA, not available. *Category variables were compared by Fisher's exact test or Chi-square test and the continuous variables were compared by Mann-Whitney test. **Data presented as median (range).

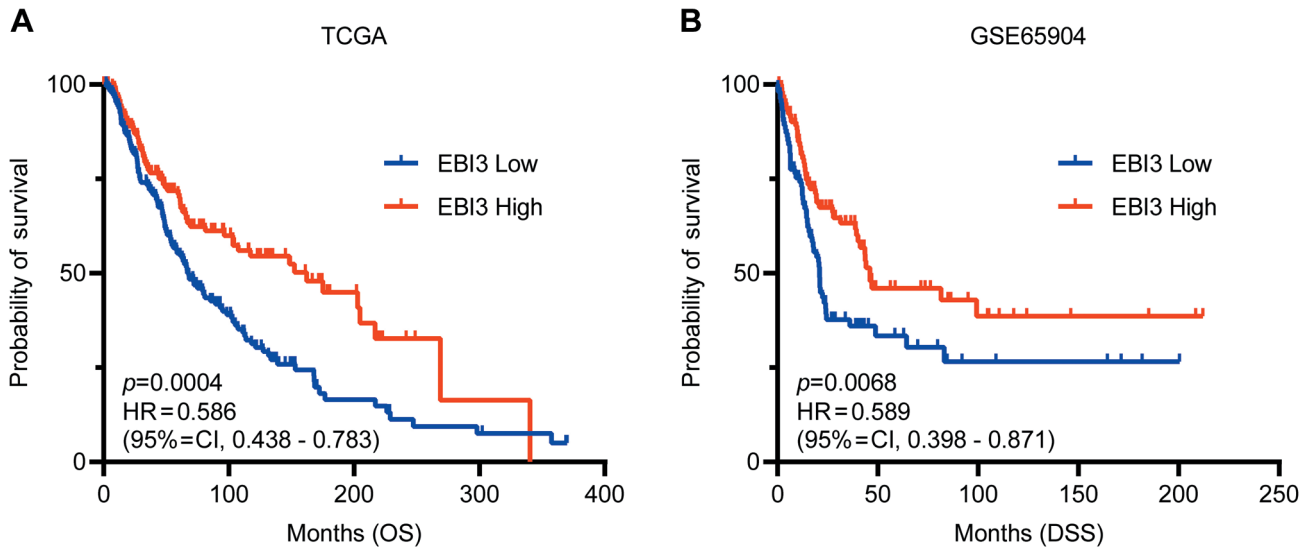

Figure 1. *Kaplan-Meier curves showing the overall survival (OS) in TCGA cohort (A) and disease-specific survival (DSS) in GSE65904 (B). The higher EBI3 mRNA expression was defined as 'EBI3 High', and the others were as 'EBI3 Low' stratified by the median level of mRNA expression. HR, Hazard ratio; CI, confidence interval.*

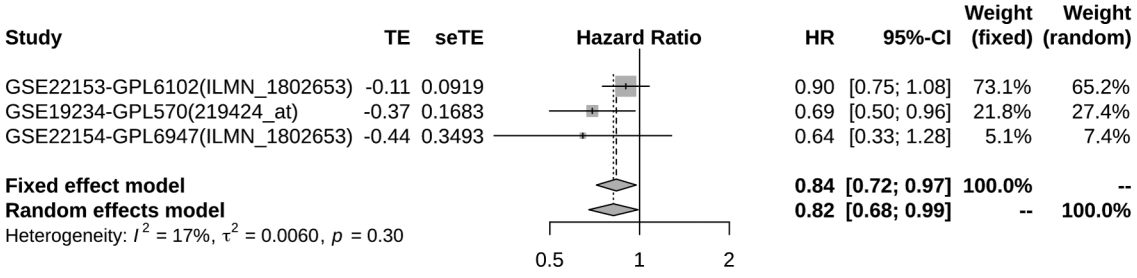

Figure 2. *Meta-survival analysis of EBI3 mRNA levels on the overall survival generated in GENT2 using GSE19234, GSE22153, and GSE22154. TE, Treatment effect; seTE, standard error of treatment estimate; HR, hazard ratio; CI, confidence interval.*

T cells/Treg ratio. In contrast, a negative correlation was observed with dendritic cells and NK cells (Figure 4B). Moreover, consistent with the *in vivo* data (26), the ratio of $CD8^+$ T cells to Tregs was significantly higher in *EBI3* high patients (Figure 4C).

Correlation of EBI3 expression with pigmentation of the melanoma tumors. Melanogenesis in melanoma cells can contribute to tumor behavior, immunosuppressive tumor microenvironment, and patients' survival (27-31). Intrigued by these studies, the correlation of melanogenesis with *EBI3* expression using the pigmentation-related gene set (24) was analyzed. *EBI3* expression in tumors was negatively correlated with the pigmentation-related gene set (Spearman's rho=–0.28) (Figure 4D).

Discussion

The current study reported a novel prognostic role of *EBI3* in metastatic melanoma associated with the distinct brisk pattern of TILs distribution and population characterized by increased $CD8^+$ T cells over Treg. To the best of the author's knowledge, the present report is the first to describe EBI3 as a new biomarker in metastatic melanoma.

This study provides clues to the potential mechanism underlying the favorable prognosis associated with TILs in *EBI3*-high patients. In the *EBI3*-high patients, the favorable spatial pattern of TILs (*i.e.*, brisk pattern) was observed. The brisk phenotype of TILs distribution is enriched with $CD8^+$ T cells compared to the non-brisk pattern (21). Consistent with this, the current study showed the increased $CD8^+$ T cells over

Table II. *Multivariate Cox regression analysis with Epstein-Barr virus-induced gene 3 (EBI3) mRNA expression levels on the overall survival on TCGA dataset.*

Variable	HR	95% CI	*p*-Value
Age at diagnosis	1.020	1.009-1.031	<0.0001
Male (ref: Female)	0.901	0.644-1.262	0.546
Stage at initial diagnosis (ref: Stage 0/I)			
II	1.019	0.649-1.600	0.935
III	1.674	1.119-2.502	0.012
IV	3.230	1.473-7.080	0.003
EBI3 mRNA level	0.737	0.652-0.834	<0.0001

TCGA: The Cancer Genome Atlas; HR, hazard ratio; CI, confidence interval; ref, reference.

regulatory T cells in *EBI3*-high tumors, in which almost three-quarters of the patients had a brisk pattern of TILs. The spatial pattern of TILs can be indicative of immune response (21). Primarily, the brisk diffuse infiltration of TILs is associated with moderate to strong immune responses (21). Thus, the gene expression of *EBI3* in the tumor can be an indicator of the nature and effectiveness of the immune response. Another evidence of *EBI3*-related favorable prognosis is the specific population of TILs, especially $CD8^+$ T cells. Previous *in vivo* experiments have shown that EBI3 is associated with increased $CD8^+$ T cells over regulatory T cells (26), consistent with the results of this study (Figure 4C). $CD8^+$ T cells are a key for antitumor immunity and prognosis in cutaneous melanoma (32, 33). TILs from EBI3–/– mice have failed to produce IFNγ (26). Regarding antitumor T-cell response, EBI3–/– mice were shown to have a phenotype of IL-27-deficiency rather than IL-35-deficiency (26). IL-27 inhibits the inducible T regulatory cells and the expression of Foxp3, CD25, and the immune checkpoint cytotoxic T lymphocyte-associated protein 4 (CTLA-4) (8, 9, 26). Contrary to IL-27, IL-35 can limit antitumor immunity by inducing Treg (7, 10). As for cytotoxic T cell (CTL) activity, IL-27 promotes CTL accumulation and inhibits tumor growth by increased CTL survival and effector functions (34-36). Taken together, the observed longer survival in EBI3-high tumors might be due to IL-27-related antitumor activity by TILs.

The role of EBI3 in oncology has been studied in breast (11), colorectal (6), lung (12), and cervical cancer (13). Interestingly, these previous studies revealed that higher EBI3 expression is an unfavorable prognostic factor, whereas we found the opposite in melanoma. Contrary to IL-27, IL-35 can explain the progression of cancers by recruiting Treg (37) or promoting tumor cell proliferation (12, 38). Therefore, further studies including preclinical models on the role of endogenous EBI3, IL-27 or IL-35 in melanoma cells

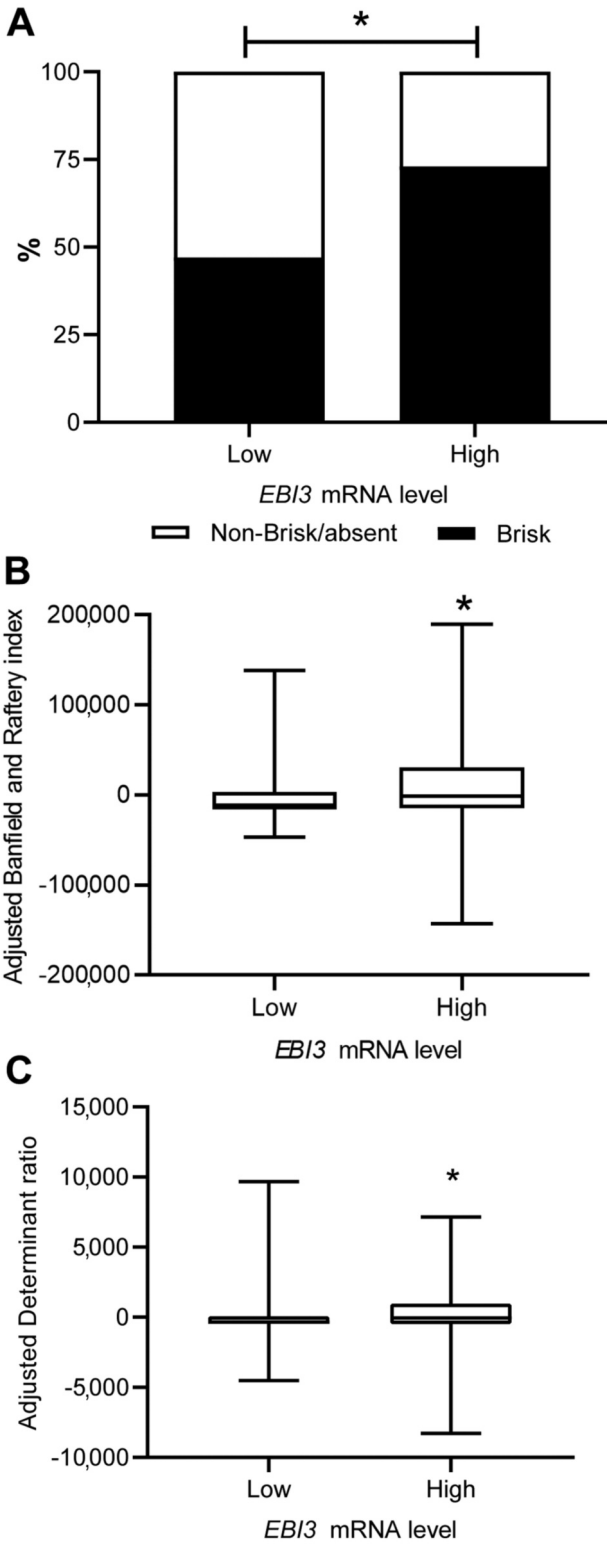

Figure 3. *Spatial profile of TILs between EBI3 high and low patients. (A) Stacked bar plot of the % of patients with spatial patterns of TILs (brisk or non-brisk/absent). *p<0.05 by Fisher's exact test; (B-C) Adjusted Banfield and Raftery index (B) and Determinant ratio (C) of clustering patterns of TILs. *p<0.05 by Mann-Whitney test.*

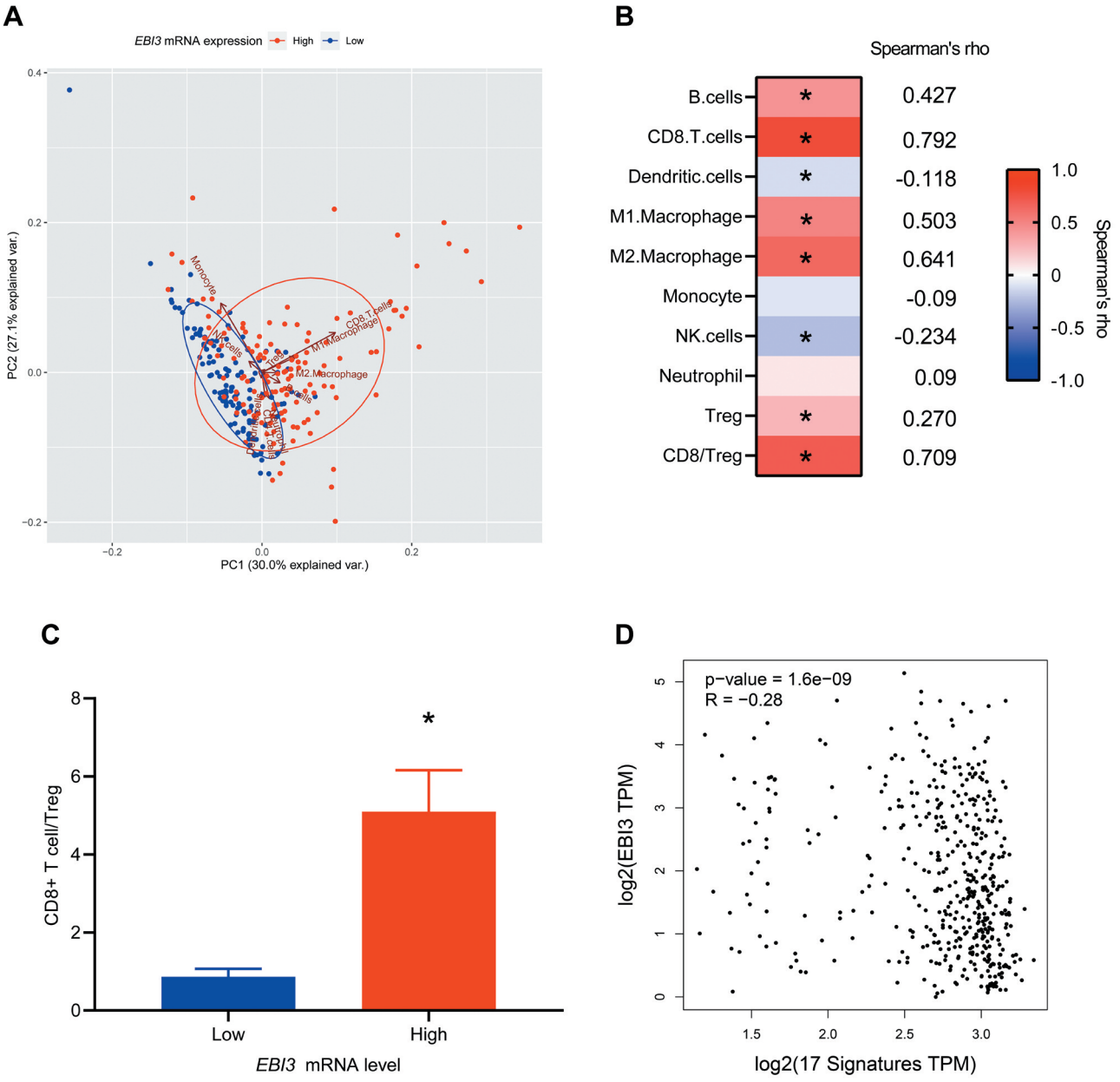

Figure 4. *(A) Principal component analysis of estimated TILs showing the shifted TILs population in EBI3-high tumors compared to EBI3-low tumors; (B) Spearman correlation of EBI3 gene expression with the TILs fractions estimated by the quanTIseq method. *p<0.05; (C) Comparison of the ratio of CD8⁺ T cell to Treg between EBI3 high and low patients. *p<0.05 by Mann-Whitney test; (D) Spearman correlation of EBI3 expression with pigmentation-related gene set. R, Spearman's rho; TPM, transcripts per million.*

or tumor microenvironment would be necessary to understand the opposite prognostic role among melanoma and the other cancers. Moreover, the current study showed the evidence of the link between *EBI3* expression and melanogenesis in tumors. Melanogenesis can affect the tumor microenvironment in an immunosuppressive way (30) and amelanotic melanoma was correlated with longer survival (27, 31). The results of this study suggest that the unfavorable prognosis in pigmented melanoma patients is partly explained by EBI3-low tumor microenvironment.

The main limitation of the current study arises from the fact that it was based only on the public database TCGA. Hence, important information such as the growth phase (vertical or not) of the tumors and metastatic status (visceral or lymph node) is

missing. Moreover, future studies are necessary to provide experimental evidence on the role of EBI3 on the tumor microenvironment in association with TILs, IL-27, and IL-35.

In conclusion, *EBI3* is a novel biomarker in metastatic melanoma with a favorable distribution pattern of increased $CD8^+$ T cells infiltration. The current study could contribute to a better understanding of a favorable tumor microenvironment of melanoma. Further studies are warranted to clarify the role of endogenous EBI3, IL-27 or IL-35 in the tumor microenvironment of melanoma.

Conflicts of Interest

None.

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