Journal for ImmunoTherapy of Cancer

BTLA promoter hypomethylation correlates with enhanced immune cell infiltration, favorable prognosis, and immunotherapy response in melanoma

Minglei Yang ,[1] Chenxi Zheng,[2] Yu Miao,[3] Cuicui Yin,[1] Longfei Tang,[1] Chongli Zhang,[1] Pu Yu,[4] Qingfang Han,[5] Yihui Ma,[6] Shenglei Li,[1] Guozhong Jiang,[1] Wencai Li,[1] Peiyi Xia[1]

To cite: Yang M, Zheng C, Miao Y, *et al*. *BTLA* promoter hypomethylation correlates with enhanced immune cell infiltration, favorable prognosis, and immunotherapy response in melanoma. *Journal for ImmunoTherapy of Cancer* 2025;**13**:e009841. doi:10.1136/jitc-2024-009841

► Additional supplemental material is published online only. To view, please visit the journal online (https://doi.org/10.1136/jitc-2024-009841).

MY, CZ and YM are joint first authors.

Accepted 17 February 2025

For numbered affiliations see end of article.

Correspondence to
Dr Minglei Yang;
yangmlei3@mail2.sysu.edu.cn

Dr Peiyi Xia;
xiapeiyi@sibcb.ac.cn

Professor Wencai Li;
liwencai@zzu.edu.cn

Dr Guozhong Jiang;
guozhongjiang@zzu.edu.cn

Professor Shenglei Li;
lslbljys@126.com

ABSTRACT

Background Immune checkpoint blockade (ICB)-based immunotherapy has significantly improved survival in advanced melanoma. However, many patients exhibit resistance to these therapies. This study examines the impact of *BTLA* promoter methylation on its expression, immune cell infiltration, and clinical outcomes, evaluating its potential as a prognostic and predictive biomarker for immunotherapy response.

Methods We analyzed methylation and gene expression data from public datasets (The Cancer Genome Atlas (TCGA), Gene Expression Omnibus (GEO)) and an in-house cohort of melanoma patients treated with ICB therapy at the First Affiliated Hospital of Zhengzhou University. We developed a quantitative methylation-specific PCR (qMSP) assay to measure methylation levels of the cg24157392 and cg03995631 CpG sites, and a targeted bisulfite sequencing assay was used to validate the accuracy of qMSP. We measured BTLA protein expression using multiplex immunofluorescence and immunohistochemical staining methods. Pearson correlation, survival analysis, and immune cell infiltration estimation were conducted to explore the associations between *BTLA* promoter methylation, mRNA and protein expression, clinical outcomes, and immune characteristics.

Results Hypomethylation at CpG sites cg24157392 and cg03995631 in the *BTLA* promoter were significantly associated with higher *BTLA* mRNA and protein expression. In the TCGA dataset, low methylation at these sites predicted longer overall survival and was validated in an independent cohort of 50 stage III/IV melanoma patients, with an area under the curve of 0.94 for predicting 5-year survival. Furthermore, *BTLA* promoter hypomethylation correlated with higher infiltration of immune cells, such as CD8+T cells, CD4+T cells, B cells, and macrophages. Additionally, low methylation at cg24157392 and cg03995631, as quantified by the qMSP assay, was significantly associated with better progression-free survival in patients treated with immune checkpoint inhibitors. These findings were further validated using GEO datasets.

Conclusions *BTLA* promoter hypomethylation serves as a significant biomarker for favorable prognosis and enhanced response to ICB therapy in melanoma. The developed qMSP assays for cg24157392 and cg03995631 accurately quantified methylation levels and demonstrated their potential for clinical application in patient stratification and personalized immunotherapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous research indicates that DNA methylation of immune checkpoint genes impacts prognosis and therapy response, but the specific epigenetic regulation of *BTLA* and its effects on immune infiltration and clinical outcomes in melanoma remain unexplored.

WHAT THIS STUDY ADDS

⇒ This study shows that *BTLA* promoter hypomethylation leads to increased *BTLA* mRNA and protein expression, higher immune cell infiltration, and better overall survival in melanoma patients, highlighting its potential as a biomarker for prognosis and immunotherapy response. Additionally, it reveals that *BTLA* promoter hypomethylation is linked to a more active tumor immune environment with enhanced antitumor immunity. The development of a quantitative methylation-specific PCR (qMSP) assay for *BTLA* promoter methylation offers a practical tool for patient stratification and personalized immunotherapy, improving clinical management of melanoma.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study could significantly impact antitumor research and clinical practice by introducing *BTLA* promoter hypomethylation as a novel biomarker for predicting prognosis and immunotherapy response in melanoma patients. It highlights the importance of epigenetic regulation in tumor-immune interactions, which could guide future research in cancer immunotherapy. Clinically, the development of the qMSP assay for measuring *BTLA* promoter methylation offers a practical tool for patient stratification and personalized treatment plans, potentially improving patient outcomes.

INTRODUCTION

With the development and clinical implementation of immune checkpoint blockade (ICB) therapy, immunotherapy has completely transformed the treatment landscape of advanced

cancers, such as melanoma, metastatic urothelial cancer, advanced non-small cell lung cancer, and colorectal cancer.[1] Checkpoint inhibitors targeting programmed death receptor-1 (PD-1) and its associated ligand (PD-L1), and cytotoxic T-lymphocyte antigen-4 (CTLA-4) as monotherapy or combination therapy with monoclonal antibodies have dramatically prolonged progression-free (PFS) and overall survival (OS) of melanoma patients.[2] However, a significant proportion of patients still show resistance to immunotherapy and cannot derive clinical benefit from it. To overcome resistance to therapy, additional immune checkpoint inhibitory receptors are being evaluated as potential targets of immunotherapy.

B and T lymphocyte attenuator (*BTLA*) gene is located on the long arm of chromosome 3. Its encoded protein belongs to the immunoglobulin superfamily, which includes PD-1, CTLA-4, CD28 and inducible T Cell costimulatory (ICOS). BTLA protein shares structural and functional similarities with CTLA-4 and PD-1,[3] including an extracellular domain, transmembrane domain, and cytoplasmic domain. Two immunoreceptor tyrosine-based inhibitory motifs are located on the cytoplasmic tail region. Herpesvirus entry mediator (HVEM) is a unique BTLA ligand. HVEM binding activates tyrosine phosphorylation and mediates the recruitment of Src homology 2 (SH2) domain containing phosphatases 1/2, leading to the inhibition of B and T cell proliferation and activation.[4] Similar to CTLA-4 and PD-1, BTLA is an immune checkpoint inhibitory receptor expressed by activated T and B lymphocytes, subsets of dendritic cells (DCs), macrophages, and natural killer cells.[5] It is involved in suppressing the antitumor immune response through HVEM-BTLA binding.[4] An ongoing clinical trial (NCT04137900) is evaluating a monoclonal antibody (TAB004) targeting BTLA for advanced malignancies in multiple centers to assess the safety, tolerability, and pharmacokinetics of TAB004. Our previous study[6] had found *BTLA* mRNA expression was associated with immunosuppressive characteristics and immunotherapy response in squamous cell lung carcinoma. Dong *et al*'s study suggests that *BTLA* mRNA expression is a reliable biomarker for prognosis and immunotherapeutic response in melanoma.[7]

The mechanism of immunotherapy response has been widely studied. Broadly, melanoma patients with high T cell infiltrations, elevated programmed death ligand-1 (PD-L1) expression,[8] high tumor mutation burden, or interferon gamma (IFNγ) signature expression are more likely to respond to immunotherapy. On the other hand, *JAK* mutations, *PTEN* loss, antigen presentation defects, WNT/β-catenin overexpression, and *PSMB8* promoter methylation[9] are associated with immunotherapy resistance, but these occur at low frequency in the melanoma patient cohort. DNA methylation is a vital epigenetic regulation mechanism that directly controls gene transcription.[10] The methylation of immune checkpoint genes including *CTLA4*,[11] *PDCD1*, *PD-L1*, *PD-L2*,[12] *TIGIT*[13] and *LAG3*[14] has been demonstrated to be predictive of prognosis and immunotherapy response in melanoma. However, the epigenetic regulation of *BTLA* expression remains unknown.

This study aims to explore the associations of DNA methylation with *BTLA* mRNA and protein expression, clinicopathological features, immunological and molecular characteristics, and clinical outcome in melanoma. To this end, we evaluated *BTLA* promoter methylation as a prognostic biomarker and as a predictive biomarker for immunotherapy response and immune cell infiltration in melanoma. Our findings provide new insights into the epigenetic regulation of *BTLA* and tumor immune microenvironment in melanoma, identifying *BTLA* promoter methylation as a potential target for developing new immunotherapy strategies.

MATERIALS AND METHODS

Public dataset collection and preprocess

Skin cutaneous melanoma (SKCM) data used in this study were partly derived from The Cancer Genome Atlas Research Network (TCGA[15]). For 470 SKCM samples from TCGA, methylation data generated by Infinium HumanMethylation450 BeadChip (Illumina, San Diego, California, USA), mRNA expression profiles generated by bulk-tissue RNA sequencing (RNA-seq) and corresponding clinicopathological data were downloaded. Furthermore, 366 metastatic SKCM samples were used as a discovery dataset; 104 primary SKCM samples as an internal validation dataset. Newell's study concurrently examined the transcriptome and methylome of pretreatment tumors from 77 patients treated with immune checkpoint inhibitors. The clinical data of these patients are available in online supplemental table S1 of the original paper.[9] For 43 tumor samples from these patients, DNA methylation array data generated by the Illumina Infinium MethylationEPIC BeadChip were retrieved from Gene Expression Omnibus (GEO) with the accession number GSE181781, which included more than 850,000 CpG sites. The expression profiles determined by RNA-seq and the clinical data of 51 pretreatment melanoma samples before treatment with immune checkpoint inhibitors were acquired from the GEO database with GSE91061. [16]RNA-seq data of 91 melanoma patients treated with immunotherapy were obtained from the European Nucleotide Archive by accession number PRJEB23709, and the clinical parameters of these patients were available in online supplemental table S2 of the original paper.[17] In addition, methylation profiles generated by Infinium HumanMethylation450 BeadChip and clinical data of 47 stage III/IV melanomas were downloaded from GEO with the accession number GSE51547.

Patients' collection at the First Affiliated Hospital of Zhengzhou University

For the in-house validation cohort, we retrospectively collected formalin-fixed, paraffin-embedded (FFPE) blocks of tumors and clinical information derived from

109 melanoma patients treated with PD-1/PD-L1 ICB at the First Affiliated Hospital of Zhengzhou University (FAHZZU). Response assessment was performed prospectively by Response Evaluation Criteria in Solid Tumors version 1.1 (RECIST 1.1).[18] Good responders were defined as those with a RECIST CR, PR, or SD for more than 6 months; poor responders had RECIST progression as best response or SD for 6 months or less. The detailed information about patient demographics and clinical characteristics including gender, age, cancer stage, and immunotherapy response is provided in online supplemental table S1.[19]

Correlation analysis between *BTLA* DNA methylation and mRNA expression

The Infinium HumanMethylation450 BeadChip (Illumina, San Diego, California, USA) was used to generate methylation data for 470 SKCM samples. The beta value data representing methylation levels included more than 450K CpG sites. The beta value data for each sample were combined into a beta-value matrix, where columns corresponded to samples and rows to CpGs. To normalize the beta-value matrix, we used the beta-mixture quantile normalization method, which was implemented using the ChAMP R package.[20] We obtained the genomic coordinates of 3 CpG sites located on the *BTLA* gene body and promoter region and saved them as a bed format file. The CpG sites and genomic structure of *BTLA* were visualized based on the bed file using the Ensembl genome browser 109. Finally, the beta values of the three CpG sites were extracted from the normalized beta-value matrix.

We obtained normalized mRNA expression data generated through RNA-Seq analysis using fragments per kilobase of transcript per million mapped reads (FPKM) from the TCGA website. The mRNA expression value of *BTLA* was extracted from this data. We performed Pearson correlation analysis between the mRNA expression of *BTLA* and the methylation levels of the three CpG sites. CpG sites located on the promoter region of *BTLA* were selected to stratify patients into high and low methylation groups based on the mean methylation value of these CpG sites.

Whole transcriptome comparison analysis

We downloaded the raw gene counts of SKCM samples from TCGA and conducted differential mRNA expression analysis between the high and low methylation groups using DESeq2.[21] Genes with an adjusted p value of less than 0.05 (Benjamini-Hochberg) and a log2 fold-change greater than 1.0 were considered to be differentially expressed.

Gene set enrichment analysis

We obtained 50 hallmark gene sets and 186 KEGG pathway signatures from the Molecular Signatures Database (MSigDB v2022.1.Hs, https://www.gsea-msigdb.org/gsea/msigdb).[22] Gene set enrichment analysis was performed using clusterProfiler[23] on the high and low promoter methylation groups to identify *BTLA* promoter methylation-related gene sets. We collected and used predictive immunotherapy signatures, such as the six-gene IFNγ signature (IFNγ-6), the related 18-gene expanded immune signature (IFNγ expanded immune 18), the effector T cell signature (effector T-cell), and the combined IFNγ Effector T-cell signature. These signatures were used to compute the single-sample gene set enrichment scores for each sample using the GSVA R package.[24]

Immune cell infiltration estimation

We used the TIMER 2.0 web application[25] to estimate the correlations between *BTLA* mRNA expression and the tumor purity, as well as the infiltration levels of B cells, CD8+T cells, CD4+T cells, macrophages, neutrophils, and DCs. We downloaded the absolute fraction data of 22 infiltrating immune cells, which was inferred by the CIBERSORT algorithm[26] based on mRNA expression profiles, from the TIMER database [25] (http://timer.cistrome.org/infiltration_estimation_for_tcga.csv.gz). Additionally, we obtained leukocyte fraction data (TCGA_all_leuk_estimate.masked.20170107.tsv) from Thorsson's study,[27] which was estimated based on DNA methylation. The percentage of tumor-infiltrating lymphocytes (TILs), evaluated by pathological images of TCGA tumors, is available in of Saltz's study.[28] Then we compared immune cell infiltration, leukocyte fraction, and TILs between high and low promoter methylation groups.

Quantitative methylation-specific PCR

Methylation analysis of the validation cohort of 109 melanoma patients from FAHZZU was conducted using bisulfite-specific quantitative real-time PCR, employing methylation-unspecific primers and probe pairs that specifically and competitively bind to methylated and unmethylated template DNA, respectively. This quantitative methylation-specific PCR (qMSP) is described in detail by Lehmann and Kreipe.[29] Tumor tissue was macrodissected from FFPE blocks for DNA extraction with a Tissue DNA Kit (Amoy Diagnostics, Xiamen, China) and subsequently underwent bisulfite conversion using the EpiArt Magnetic DNA Methylation Bisulfite Kit (Nanjing Vazyme Biotech) according to the manufacturer's protocol. We developed the qMSP assay targeting the CpG site as probed by Illumina HumanMethylation450 BeadChip bead cg24157392 and cg03995631 (figure 1). Uncalibrated methylation levels, approximately considered percent methylation, were computed using cycle threshold (CT) values obtained from the probes specifically binding to methylated ($CT_{methylated}$) and unmethylated ($CT_{unmethylated}$) DNA, respectively (methylation [%]=$100\%/(1+2^{CTmethylated-CTunmethylated})$). We performed 20 µL triplicate PCR reactions using a buffer composition containing 20 ng bisulfite-converted DNA (quantified via UV-VIS spectrophotometry) and 0.2 µM each probe and 0.2 µM each primer (qMSP for cg24157392 CpG site forward primer: aggtaggtgaatattatgttttaatttgg, reverse primer: ataatcrtaaataaaaccaaactattacatt, probe

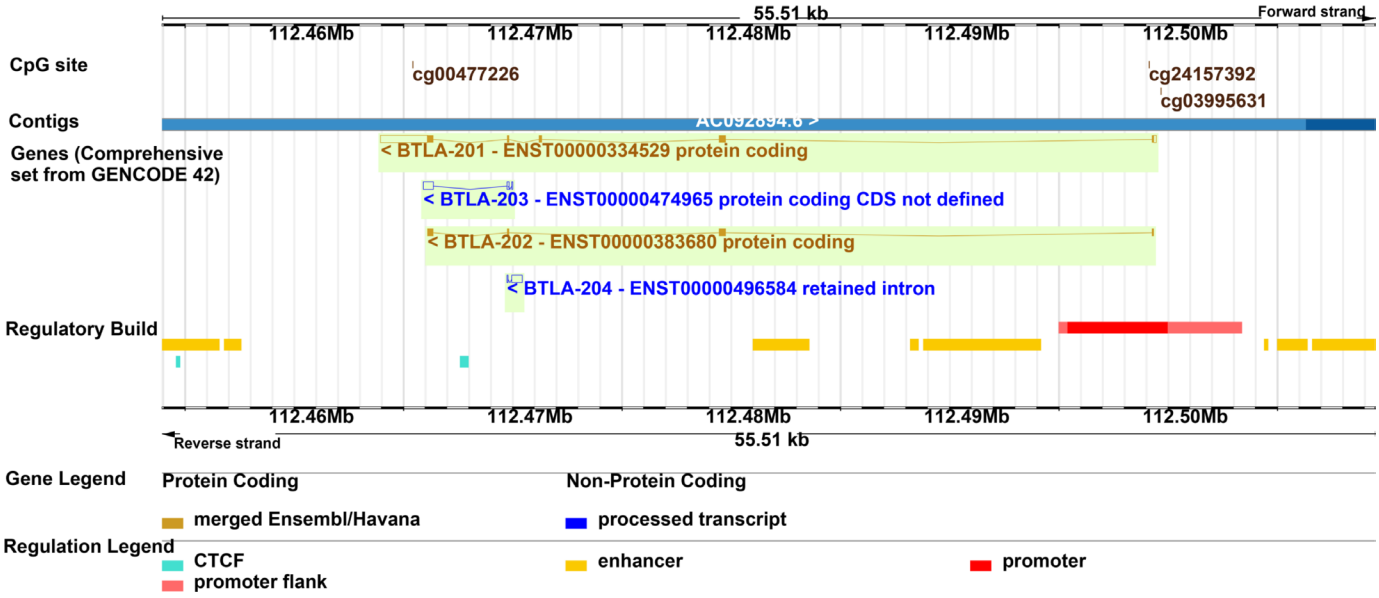

Figure 1 Overview of CpG sites detected by Infinium HumanMethylation450 BeadChip and genomic structure of *BTLA*. The illustration (modified) exported from www.ensembl.org (release 113)[36] showing chromosome 3: 112453966-112509472 region, including the *BTLA* gene and regulatory elements (promoter, promoter flank, enhancer, CTCF binding sites). The two CpG sites (cg24157392 and cg03995631) were located on the promoter region, and the remaining CpG site (cg00477226) was located on exon 5.

methylated: 6-FAM-cttaccaaaaac-MGB-1, probe unmethylated: HEX-cttaccgaaaac-BHQ-1; qMSP for cg03995631 CpG site forward primer: ggttggttgtttatttttaaatattgtg, reverse primer: aaaaaacctcaacctaaaacaaaaac, probe methylated: 6-FAM-taataaaacaaaaaa-MGB-1, probe unmethylated: HEX-taataaaacgaaaaaa-BHQ-1). qMSP was carried out using a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA) with the following temperature profile: 20 min at 95°C and 45 cycles with 50 s at 95°C, 50 s at 57°C and 50 s at 72°C.

Targeted bisulfite sequencing assay

DNA extraction and bisulfite conversion were performed as previously described.[29] Based on the genomic coordinate of cg24157392 and cg03995631 CpG sites, we designed two paired primers (cg24157392 targeted bisulfite sequencing assay forward primer: attgatgygatgtttatgattaggtag, reverse primer: atcttaaaaacaacattctccct; cg03995631 targeted bisulfite sequencing assay forward primer: aagagtttggatgattttgtgaa, reverse primer: caaaaaaaacctcaacctaaaac) to detect it. The net-PCR was performed first to amplify the targeted DNA sequence. Then, the designed DNA fragments were sequenced by Illumina Hiseq 2000. BSseeker2, one of the most commonly used tools for analyzing the bisulfite sequencing results, was applied in our study for mapping bisulfite-treated reads as well as for methylation calling. After calling methylation, we obtained the bisulfite conversion rate for each sample, and samples with bisulfite conversion rates <98% were first filtered out. After the preliminary analysis, we then calculated the average coverage as well as the missing rate for each CpG site. The CpG sites with average coverage less than 20× and/or with missing rate >0.20 were further filtered out. In addition, the samples with a missing rate >0.30 were filtered out.

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was used to quantify *BTLA* mRNA expression levels in 109 melanoma samples of the FAHZZU cohort. RNA extraction was performed using a FFPE DNA/RNA extraction Kit (AmoyDx, Xiamen, China) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA using the HiScript III RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China) according to the manufacturer's instructions. The qRT-PCR assay was performed in 20 µL volumes using Taq Pro Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) containing 20 ng cDNA and 0.2 µM each primer (*BTLA* forward primer: tgggtcataccgctgttctgca, *BTLA* reverse primer: ctgcttgccatttcgtccttgg; *DNMT3B* forward primer: taacaacggcaaagaccgaggg, *DNMT3B* reverse primer: tcctgccacaagacaaacagcc; *DNMT3A* forward primer: cctcttcgttggaggaatgtgc, *DNMT3A* reverse primer: gtttccgcacatgagcacctca). The housekeeping genes *ACTB* and *GAPDH* were used as references for normalization (*ACTB* forward primer: atgtggccgaggactttgatt, *ACTB* reverse primer: agtggggtggcttttaggatg; *GAPDH* forward primer: tgcaccaccaactgcttagc, *GAPDH* reverse primer: ggcatggactgtggtcatgag). qRT-PCR was carried out using a Bio-Rad CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, California, USA) with the following temperature profile: 30 s at 95°C and 40 cycles with 10 s at 95°C and 30 s at 60°C. Relative *BTLA* mRNA expression levels were calculated using the ΔCT method.

Multiplex immunofluorescence and image acquisition

Multiplex immunofluorescence staining was performed with a three-color fluorescence immunohistochemistry kit (PhenoVision Bio) following the manufacturer's instruction. In short, tissue sections went through deparaffinization, rehydration, and were transferred into citrate buffer pH6.0 for antigenic repair. The sections were then washed and incubated with H2O2 and blocking reagents for 10 min, respectively. Primary antibody (BTLA and CD45RO from Abcam and ZSbio, respectively) was applied on each section for 30 min at RT. Bound primary antibodies were detected using PVB anti-Rb/Mm-HRP detection, followed by PVB tyramide signal amplification fluorophore (PVB 520 and PVB570) for 10 min. The slides were counterstained with 4′,6-diamidino-2-phenylindole (PhenoVision Bio) and cover-slipped. The slides were scanned using the PhenoImage HT system (Akoya Biosciences) at 200× magnification.

Immunohistochemical analysis for BTLA

FFPE tissue sections of 4 µm thickness were stained for BTLA with an anti-human BTLA rabbit monoclonal antibody (anti-CD272/BTLA, ab212089, Abcam). The immunohistochemical (IHC) staining was performed on the Roche (Ventana BenchMark ULTRA) immunohistochemistry/ISH Automatic Staining Module. The detection system used was the Roche Ventana UV AP-Red kit. The consecutive steps were performed with the following (1) EZ deparaffinization, 12 min; (2) CC1, 64 min; (3) incubation with primary antibody (BTLA), 48 min; (4) UV red univ mult, 12 min; (5) UV red enhancer, 4 min; (6) UV fast red A/UV red naphthol, 8 minutes; (7) UV fast red B, 8 min; (8) counterstain with Mayer hematoxylin, 12 min; (9) postcounterstain with bluing reagent, 4 min. For IHC staining images, we used the semiquantitative method H-score to evaluate the expression of BTLA protein. The semiquantitative H-score coefficient was calculated using the following formula: percentage of weakly staining cells+percentage of moderately staining cells×2+percentage of strongly staining cells×3.[30]

STATISTICAL ANALYSIS

All statistical analyses were performed in the R environment. Pearson correlation analysis was used to calculate the correlations between two variables, using the 'cor.test' function in R. Comparisons of continuous values between two groups were assessed with the Wilcoxon-Mann-Whitney U test. The Kaplan-Meier estimate and log-rank tests were used for the survival analysis of OS and PFS. We employed a multivariate Cox proportional hazard model to investigate the association between the combination of *BTLA* promoter methylation, clinical tumor stage, age, and gender with survival.

RESULTS

Association of *BTLA* mRNA expression and methylation

DNA methylation is a chemical modification and an important epigenetic regulation of mRNA expression. Hypermethylation of DNA functional elements, including promoters and enhancers, often results in transcriptional silencing.[31] To investigate whether DNA methylation regulated *BTLA* mRNA expression, we first correlated the methylation level of 3 CpG sites within the *BTLA* gene with the mRNA expression value of this gene among 366 metastatic SKCM samples from TCGA. The methylation of these CpG sites was detected by Illumina Infinium HumanMethylation450 BeadChip. According to the genomic coordinate from Illumina Infinium HumanMethylation450 BeadChip, we used Ensembl genome browser 113[32] to map the 3 CpG sites on the genomic organization of the *BTLA* gene, which is located on the reverse strand of chromosome 3. *BTLA* has four transcripts including two protein-coding transcripts (*BTLA*-201 and *BTLA*-202) and two non-protein-coding transcripts (*BTLA*-203 and *BTLA*-204) (figure 1). The transcription start sites of the two protein-coding transcripts were close to each other. Two CpG sites (cg24157392 and cg03995631) were located in the promoter region of the *BTLA* gene, while cg00477226 was located in exon 5 of the *BTLA*-201 transcript.

Pearson correlation analysis showed significant inverse correlations between *BTLA* mRNA expression and DNA methylation of the 2 CpG sites including cg03995631 (figure 2A, R=−0.74, p<0.001) and cg24157392 (figure 2B, R=−0.81, p<0.001). However, the methylation level of cg00477226 (figure 2C, R=0.018, p=0.73) located on exon 5 did not have a significant correlation with mRNA expression of *BTLA*. Interestingly, we observed a notable consistency in methylation levels between cg24157392 and cg03995631 located on the promoter region (figure 2D, R=0.87, p<0.001). Then the mean beta value of these two CpG sites was defined as promoter methylation. As expected, the promoter methylation level was reversely correlated with *BTLA* mRNA expression (figure 2E, R=−0.8, p<0.001). Notably, the normalized mRNA expression of *BTLA* (log2(FPKM+1)) in most samples was significantly low, typically less than 1, which corresponded to a high methylation level of the *BTLA* promoter (figure 2E). These results supported the notion of an epigenetic regulation of *BTLA* mRNA expression via promoter methylation.

Promoter hypomethylation of *BTLA* was correlated with favorable prognosis

Of 366 metastatic SKCM patients, 356 patients have OS times. To investigate whether *BTLA* promoter methylation and mRNA expression could predict the prognosis of melanoma, we stratified the 356 metastatic SKCMs into two groups (high and low groups) based on the median beta values of the two CpG sites (cg03995631: 0.8554, cg24157392: 0.8603) and the median FPKM of *BTLA* (0.3116), respectively, and correlated the two groups with

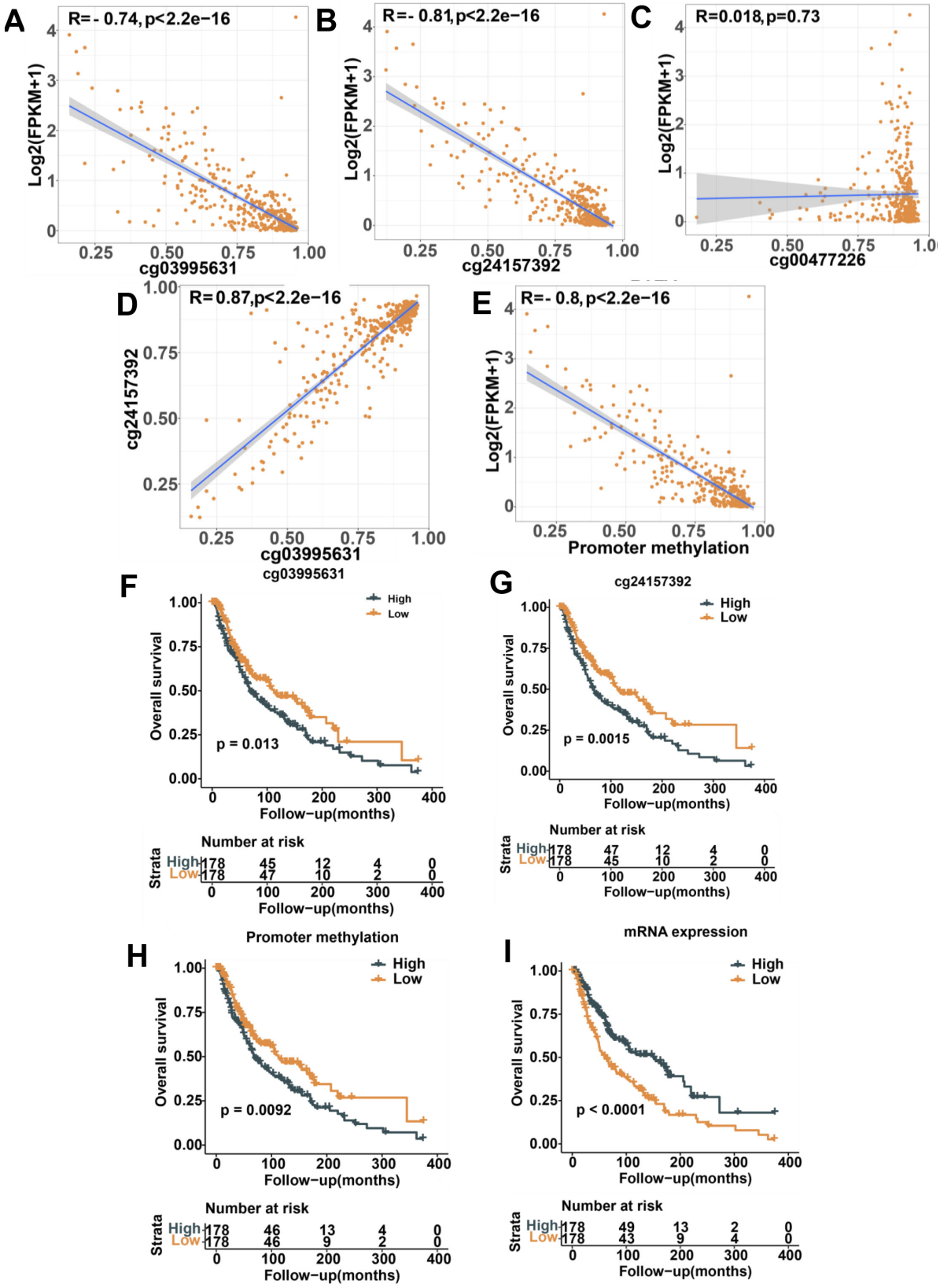

Figure 2 Correlations of DNA methylation with *BTLA* mRNA expression and prognosis in 366 metastatic melanoma patients. (A–C) Pearson correlations of beta value of CpG sites (cg03995631, cg24157392, and cg0047726) with *BTLA* mRNA expression. (D) Methylation correlation between cg24157392 and cg03995631. (E) The correlation between promoter methylation level (defined as the mean beta value of cg03995631 and cg24157392) and *BTLA* mRNA expression. (F–G) Kaplan-Meier curves of overall survival in melanoma patients stratified according to median beta values of cg03995631 and cg24157392. (H) Kaplan-Meier curves of overall survival in these patients stratified according to promoter methylation level defined by mean Beta value of two CpG sites. (I) Kaplan-Meier curves of overall survival in these patients stratified according to *BTLA* mRNA expression.

OS. We observed that hypomethylation of cg03995631 (figure 2F, p=0.013) and cg24157392 (figure 2G, p=0.0015) was significantly associated with prolonged OS time. Based on the median value of *BTLA* promoter methylation (0.8547), defined by the mean methylation level of the two CpG sites, we also observed that the melanoma patients belonging to the low methylation group have longer OS (p=0.0092) than those belonging to the high methylation group (figure 2H). We had observed that the promoter hypomethylation of *BTLA* mirrored high mRNA expression levels of this gene. As expected, we also found that increased mRNA expression of *BTLA* was associated with prolonged OS time (figure 2I, p<0.0001). Multivariate survival analysis using the Cox regression model indicated that the *BTLA* promoter hypomethylation was retained as an independent prognostic factor for OS (p=0.005) in the metastatic SKCM (online supplemental figure 1). Overall, *BTLA* promoter hypomethylation correlates with increased mRNA expression, which can be used as predictive biomarkers of favorable prognosis.

Promoter hypomethylation was associated with enhanced immune cell infiltration and elevated mRNA expression level of signatures related to immunotherapy response

Tumor-infiltrating immune cells have been identified as independent factors for predicting prognosis.[33] As a result, we examined the influence of *BTLA* promoter methylation and mRNA expression on immune cell infiltration in the tumor microenvironment (TME) of metastatic SKCM. The TIMMER 2.0 web application analysis revealed an inverse relationship between *BTLA* mRNA expression and tumor purity (figure 3A), suggesting *BTLA* might play a crucial role in suppressing tumor growth. In terms of immune cells, *BTLA* mRNA expression positively correlated with the infiltration levels of B cells, CD8+T cells, CD4+T cells, macrophages, neutrophils, and DCs (figure 3A), implying that *BTLA* could affect lymphocyte infiltration. To investigate if *BTLA* promoter methylation was also connected to immune cell infiltration, we compared the infiltration levels of 22 immune cell types, as estimated by CIBERSORT,[26] between high and low *BTLA* promoter methylation groups in metastatic SKCM samples. We discovered that 15 immune cell types were significantly more abundant in the low promoter methylation group (figure 3B). Among these, CD8+T cells, memory B cells, naïve B cells, and M1 macrophages have been previously linked to a favorable prognosis for SKCM.[33 34] Additionally, both the leukocyte fraction (figure 3C) calculated from methylation data and the TIL percentage (figure 3D) assessed by pathological images were higher in the low promoter methylation group. The cytolytic activity score, defined as the geometric mean of *GZMA* and *PRF1* mRNA expression values and associated with antitumor immune response and prognosis,[35] was also higher in the low promoter methylation group (figure 3E). These findings suggest that hypomethylation of the *BTLA* promoter and elevated *BTLA* mRNA expression are linked to increased immune cell infiltration levels, which subsequently impact prognosis.

To uncover the transcriptomic molecular features linked to *BTLA* promoter methylation, we divided 366 metastatic SKCM cases into high and low promoter methylation groups based on mean beta value of two CpG sites (cg24157392 and cg03995631) and performed a differential expression analysis between the two groups. We found a notable increase in the mRNA expression of 2786 genes in the low methylation group, including 8 immune checkpoint genes such as *TIGIT*, *PDCD1*, *LGA3*, *HAVCR2*, *CD274*, *C10orf54*, and *SIGLEC7* (online supplemental table S2 and figure 3F). Unbiased gene set enrichment analysis revealed 8 hallmark gene sets and 41 KEGG pathways that were significantly overrepresented in the low methylation group (online supplemental table S3). Of note, hallmark gene sets and KEGG pathways linked to anti-cancer immune responses, such as the inflammatory response, interferon alpha response, interferon gamma, antigen processing and presentation, and others, were significantly enriched in the low methylation group (figure 3G). We also discovered a strong inverse relationship between the methylation level of two CpG sites and the mRNA expression of immune checkpoint genes. *BTLA* mRNA expression showed significant positive correlation with the expression of these genes, implying that *BTLA* promoter methylation might regulate mRNA expression and influence antitumor immune responses (figure 3H). Furthermore, we examined the expression of four gene sets previously associated with immunotherapy response and found that these genes were significantly more expressed in the low methylation group. The enrichment scores of these gene sets, such as a 6-gene IFNγ signature, a related 18-gene IFNγ signature, an effector T cell signature, and a combined IFNγ/effector T cell signature were notably higher in the low methylation group compared with the high methylation group (figure 3I,L).

BTLA promoter hypomethylation correlated with its mRNA expression, immune cell infiltration, and prognosis in primary melanomas

To validate the links between *BTLA* promoter methylation and biological as well as immune characteristics in SKCM, we carried out identical analyses on 104 primary SKCM samples sourced from the TCGA. In line with metastatic SKCM, we also discovered that the methylation levels of cg03995631 (R=−0.48, p<0.0001, online supplemental figure 2A) and cg24157392 (R=−0.56, p<0.0001, online supplemental figure 2B) displayed significant inverse correlations with *BTLA* mRNA expression. Furthermore, there was a marked positive correlation in the methylation level between cg03995631 and cg24157392 (R=0.67, p<0.0001, online supplemental figure 2C). As anticipated, the average methylation level of the two CpG sites located in the promoter region exhibited a considerable inverse correlation with *BTLA* mRNA expression (R=−0.56, p<0.0001, online supplemental figure 2D).

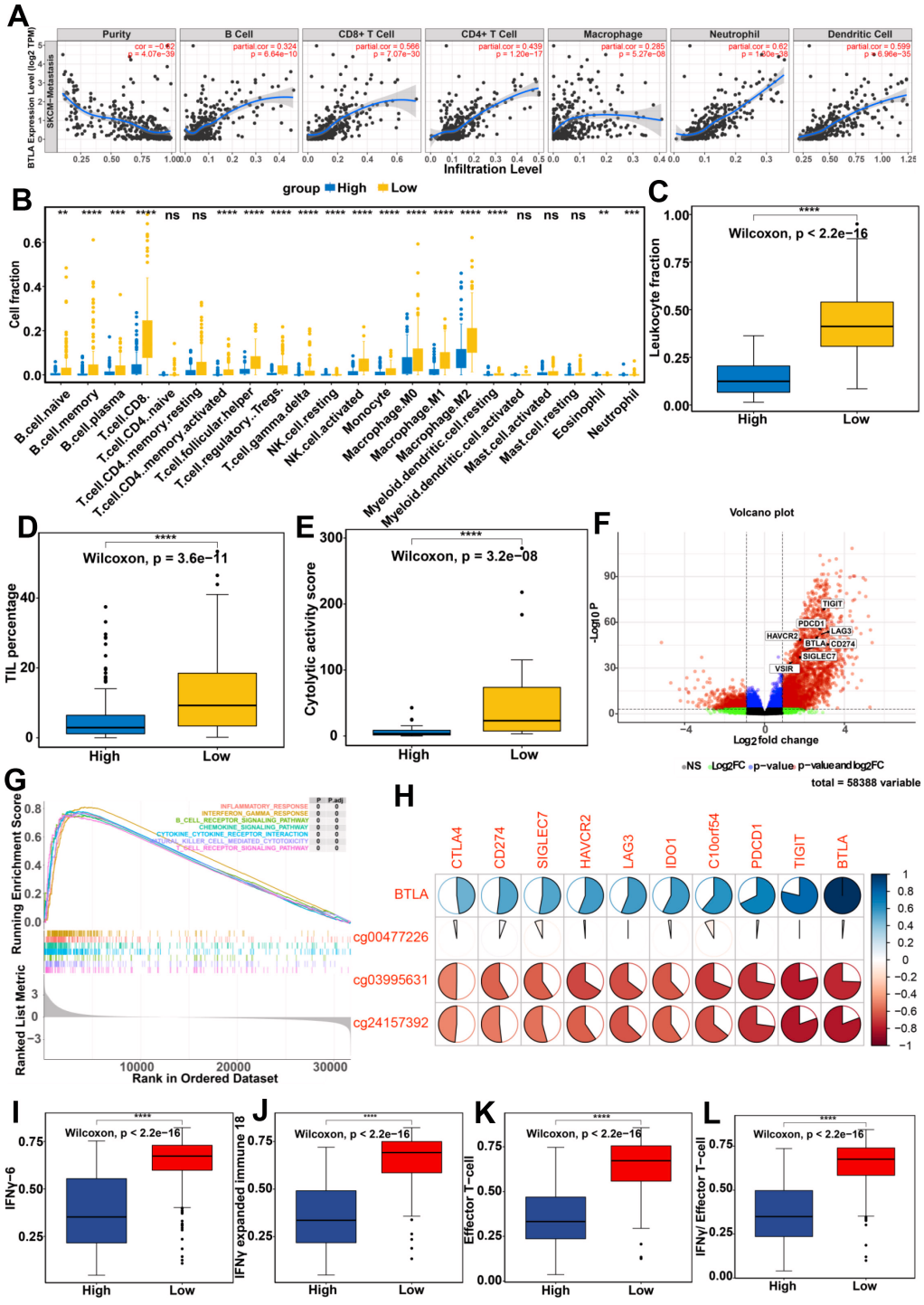

Figure 3 Correlations of *BTLA* promoter hypomethylation with high immune cell infiltration and transcriptomic alterations of molecules and pathways in metastatic melanomas. (A) The correlations of *BTLA* mRNA expression with tumor purity and the infiltration levels of immune cells (B cell, CD8+T cell, CD4+T cell, macrophages, neutrophil and dendritic cells). (B) The comparison of the absolute fraction of TME cells between the high and low promoter methylation groups. (C–E) Box plots show the differences in leukocyte fraction, TIL percentage and cytolytic activity between the two groups. All statistical differences of two groups were compared by Wilcoxon rank-sum test; **p<0.01, ***p<0.001, ****p<0.0001. (F) Volcano showing differentially expressed genes between low and high promoter methylation groups; genes with log2 fold change >1 and p<0.05 are considered to be upregulated in low promoter methylation melanomas. (G) Gene set enrichment analysis plot of hallmark gene sets and pathways enriched (FDR<0.05) in the low promoter methylation group. (H) The correlations between the methylation level of two CpG sites and mRNA expression of immune checkpoint genes. (I–L) Box plots of gene set enrichment scores. Box plots show the median, first and third quartiles, and the whiskers extend to 1.5 times the IQR and p values were calculated using two-sided Wilcoxon rank sum. (I) IFNγ–6. (J) IFNγ expanded immune 18. (K) Effector T cell. (L) IFNγ/Effector T cell. All statistical differences of two groups were compared by Wilcoxon rank-sum test; **p<0.01, ***p<0.001, ****p<0.0001. TIL, tumor-infiltrating lymphocyte; TME, tumor microenvironment.

Using the average methylation level of the two CpG sites as a basis, we partitioned the 104 primary SKCM samples into high and low promoter methylation groups. Parallel to metastatic SKCM, we observed that the cell fractions of various immune cell types, including CD8+T cells, activated memory CD4+T cells, T follicular helper cells, activated NK cells, and M1 macrophages, were noticeably higher in the low methylation group compared with the high methylation group (online supplemental figure 2E). The leukocyte fraction deduced from DNA methylation (online supplemental figure 2F) and the cytolytic activity score (online supplemental figure 2G) were also higher in the low methylation group. When we looked at the prognostic value of *BTLA* promoter methylation, high methylation level of cg03995631 (p=0.0023, online supplemental figure 2H) was significantly associated with decreased PFS in 104 primary SKCM patients. In addition, the patients with high methylation levels of cg03995631 tended to have poor PFS (online supplemental figure 2I). Subsequently, hypermethylation of the *BTLA* promoter defined by mean beta value of cg03995631 and cg03995631 was significantly correlated with poor PFS (online supplemental figure 2J). In summary, our research, based on 104 primary SKCM samples, further confirms the substantial inverse correlations of *BTLA* promoter methylation with *BTLA* mRNA expression, immune cell infiltration, and prognosis.

Validation of predictive value of promoter methylation for OS in an independent cohort

An independent cohort, which included 47 stage III/IV melanoma tumors from GSE51547, was used to further validate the prognostic value of *BTLA* promoter methylation. We divided 47 patients into high methylation and low methylation groups based on the median values of cg03995631, cg24157392, and promoter methylation beta values (0.878, 0.896, and 0.869, respectively). Survival analysis based on the methylation levels of cg03995631 (figure 4A, p=0.016), cg24157392 (figure 4B, p=0.00022) and promoter (figure 4C, p=0.0021) indicated that patients with *BTLA* promoter hypomethylation showed significantly prolonged OS time. We also performed area under the curve (AUC) analysis for the receiver operating characteristic to compare sensitivity and specificity for prediction of death at different cut-off times (2, 3, and 5 years) by the methylation level of cg03995631 (figure 4D), cg24157392 (figure 4E) and *BTLA* promoter (figure 4F). Then we found that AUC of 5 year survival reached 0.88 (cg03995631), 0.94 (cg24157392), and 0.92 (*BTLA* promoter), respectively. Guo *et al* developed a four-DNA methylation biomarker for predicting OS of patients with cutaneous melanoma. However, the four-DNA methylation biomarker only achieved an AUC of 0.708 for predicting 5-year survival in the cohort from GSE51547. Compared with a four-DNA methylation biomarker identified by Guo *et al*[36], *BTLA* promoter methylation showed superior performance for predicting OS in melanoma patients.

Associations of *BTLA* promoter methylation and mRNA expression with clinical outcome in melanoma patients treated with immunotherapy

In Newell's study, the pretreatment tumor specimens of 35 melanoma patients who received ICB therapy concurrently underwent RNA-seq and methylation profiling.[9] In line with TCGA datasets, Pearson correlation analysis validated that the methylation levels of cg24157392 (figure 4G, R=−0.83, p=7.8e−10) and cg03995631 (figure 4H, R=−0.83, p=7.7e−10) showed significant inverse correlation with *BTLA* mRNA expression. Newell's study performed methylation profiles on the tumor specimens of 43 melanoma patients, and survival analysis based on the median methylation values of cg24157392 (0.8643) and cg03995631 (0.8752) indicated that low methylation levels of cg24157392 (figure 4I, p=0.022) and cg03995631 (figure 4J, p=0.088) located on the *BTLA* promoter region were associated with prolonged PFS. In addition, we validated the prognostic value of *BTLA* mRNA expression in two independent melanoma cohorts treated with ICB therapy. In the 51 melanoma patients from the GSE91061 dataset, the patients with high *BTLA* mRNA expression had significantly prolonged progress free survival after receiving ICB therapy (online supplemental figure 3A, p=0.0037). Similarly, in the 91 melanoma patients from PRJEB23709, patients with high *BTLA* mRNA expression had significantly prolonged OS after receiving ICB therapy (online supplemental figure 3B, p=0.0061).

Validation of the association of cg24157392 methylation with immunotherapy response in the FAHZZU cohort

The metastatic melanoma patients receiving ICB therapy from the FAHZZU cohort (N=109) were studied to test *BTLA* promoter methylation as a predictive biomarker for disease progression after treatment. First, we correlated the methylation levels of cg24157392 and cg03995631 CpG sites with mRNA expression of *BTLA* in the FAHZZU cohort. Pearson correlation analysis further validated hypomethylation of cg24157392 (R=−0.31, p<0.0001) and cg03995631 (R=−0.34, p=0.00022) CpG sites was significantly associated with elevated mRNA expression of *BTLA* (figure 4K). We correlated PFS with methylation levels in pretreatment samples measured by qMSP targeting the cg24157392 and cg03995631 CpG sites. When we further dichotomized the methylation data applying optimized cut-off values (cg24157392: 0.94; cg03995631: 0.8) provided in online supplemental table S1. Kaplan-Meier survival curves demonstrated a better PFS for melanoma patients with hypomethylated cg24157392 (p=0.012) and cg03995631 (p=0.035) (figure 4L). To further validate the accuracy of qMSP, we correlated the methylation levels of cg24157392 and cg03995631 CpG sites calculated by targeted bisulfite sequencing assay with those by qMSP. Pearson correlation analysis indicated that the two quantitative methods showed remarkable consistency for cg24157392 (R=0.92, p<0.0001) and cg03995631 (R=0.91, p<0.0001) CpG sites (figure 4M). Comparative

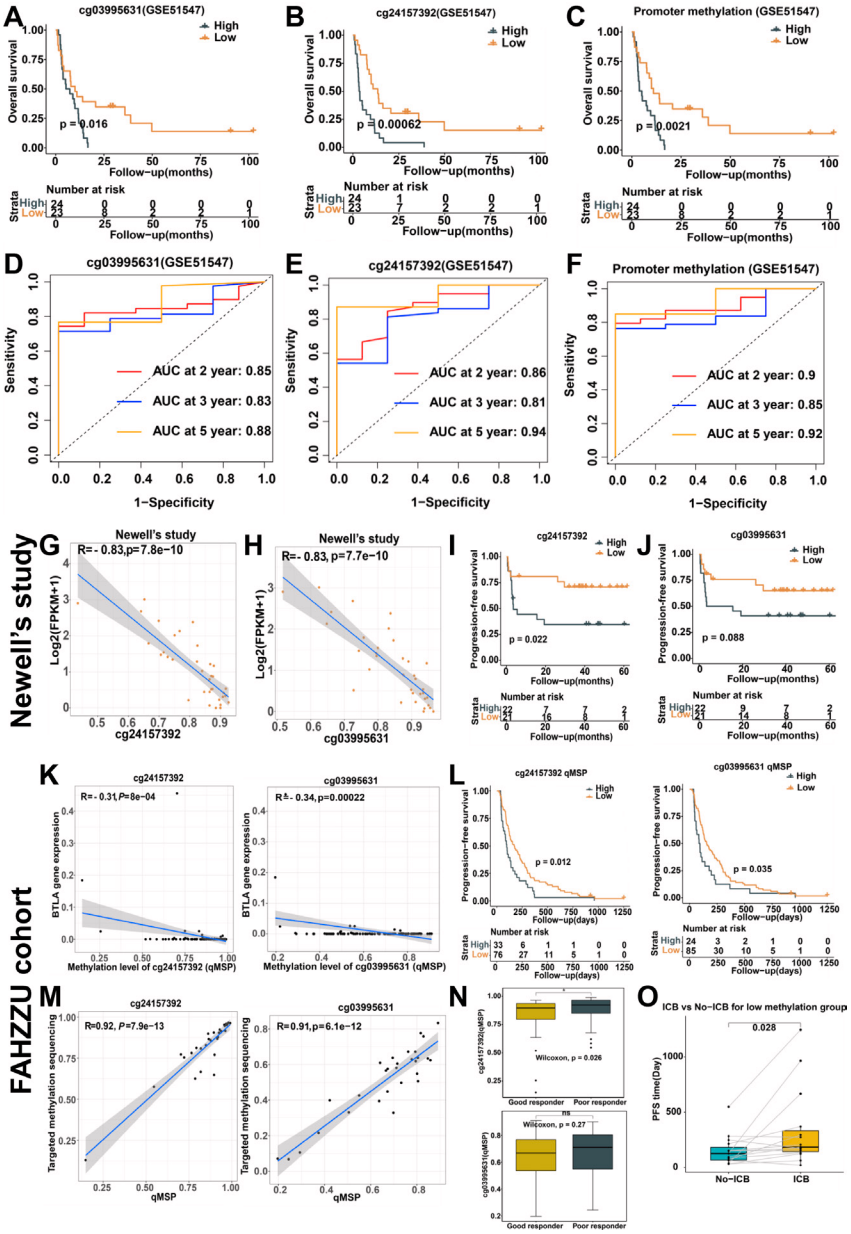

Figure 4 Validation of correlations of *BTLA* methylation with prognosis and mRNA expression in independent melanoma cohorts and the FAHZZU cohort treated with immune checkpoint blockade (ICB) therapy. (A–C) The OS analysis based on cg03995631 (A), cg24157392 (B) and promoter (C) methylation level. (D–F) ROC analyses of the cg03995631 (D), cg24157392 (E), and promoter (F) methylation level in predicting 5 years OS. (G–H) Correlations of cg24157392 (G) and cg03995631 (H) methylation with *BTLA* mRNA expression in the melanoma cohort treated with immunotherapy from Newell's study. (I–J) Progression-free survival analysis based on cg24157392 (I) and cg03995631 (J) methylation level in the cohort from Newell's study. (K) Scatter plot showing the negative correlation between *BTLA* mRNA expression and methylation level of the cg24157392 site as measured by qMSP. Lower methylation levels are associated with higher mRNA expression. (L) Kaplan-Meier survival curve depicting progression-free survival in melanoma patients stratified by high and low methylation levels of cg24157392 as measured by qMSP. Patients with low methylation levels (n=76) had significantly longer progression-free survival compared with those with high methylation levels (n=33) (p=0.012). (M) Scatter plot demonstrating the strong positive correlation between methylation levels of cg24157392 measured by qMSP and targeted methylation sequencing (R=0.92, p=7.9e–13), validating the accuracy of qMSP for assessing methylation at this site. (N) Scatter plot showing the negative correlation between *BTLA* mRNA expression and methylation level of the cg03995631 site as measured by qMSP. Lower methylation levels are associated with higher mRNA expression. (O) Kaplan-Meier survival curve depicting progression-free survival in melanoma patients stratified by high and low methylation levels of cg03995631 as measured by qMSP. Patients with low methylation levels (n=85) had significantly longer progression-free survival compared with those with high methylation levels (n=24) (p=0.035). (P) Scatter plot demonstrating the strong positive correlation between methylation levels of cg03995631 measured by qMSP and targeted methylation sequencing (R=0.91, p=6.1e–12), validating the accuracy of qMSP for assessing methylation at this site. AUC, area under the curve; FAHZZU, First Affiliated Hospital of Zhengzhou University; OS, overall survival; qMSP, quantitative methylation-specific PCR; ROC, receiver operating characteristic.

analysis showed that for the cg24157392 site (p=0.026), good responders exhibited significantly lower methylation levels, while for the cg03995631 site (p=0.27), the methylation levels of good responders tended to be lower than those of poor responders (figure 4N). We collected PFS data for 15 patients in the low methylation group who received other treatments (such as targeted therapy or chemotherapy) prior to ICB therapy (online supplemental table S1). A paired t-test was used to compare the PFS time between ICB and no-ICB therapy. The results showed that these patients had a longer PFS for ICB therapy (figure 4O, p=0.028). Overall, the qMSP assay developed in this study for targeting cg24157392 and cg03995631 could accurately quantify the methylation levels of the two CpG sites, and hypomethylation of cg24157392 and cg03995631 was significantly associated with prolonged PFS of patients treated with ICB therapy.

BTLA promoter methylation was associated with its protein expression and immune cell infiltration in the FAHZZU cohort

Previous studies have shown that BTLA is expressed on T cells.[3] CD45RO is a marker for human memory T cells.[37] To confirm the expression of BTLA on T cells in melanoma, a panel of multiplex immunofluorescence staining with BTLA and CD45RO was performed on three melanoma FFPE tissue sections. Then we observed the colocalization of BLTA (yellow signal) and CD45RO (red signal) in some cells (figure 5A and online supplemental figure 4A and B), demonstrating that BTLA was expressed on T lymphocytes in melanoma. We performed H&E staining and BTLA IHC staining on tumor samples of 100 melanoma patients from the FAHZZU cohort and evaluated BTLA protein expression level with H-score. We found that tumors in the low promoter methylation group had more lymphocyte infiltration (figure 5B and online supplemental figure 4C) and a higher H-score (figure 5C, p=0.022). Pearson correlation analysis also showed a significant negative correlation between the methylation level of the *BTLA* promoter and H-score (figure 5D, R=−0.69, p<0.001), suggesting that the methylation level of the *BTLA* promoter is negatively correlated with the BTLA protein level. The Systemic Immune-Inflammation Index (SII), as an evaluation index of systemic inflammatory response, has been confirmed to be related to the prognosis.[38] It is calculated using a formula that takes into account the levels of certain immune system markers in the blood. The calculation formula is platelets×neutrophils/lymphocytes.[38] We calculated the SII levels for a total of 109 melanoma patients from the FAHZZU cohort (online supplemental table S1) and compared the differences between the low and high methylation groups. We found that the low promoter methylation group had a lower SII (figure 5E), suggesting that the low methylation group may have a better response to immunotherapy. Based on the SII threshold of 330 provided by the authors in the original study,[38] the FAHZZU cohort was divided into high and low SII groups. We further analyzed the correlation between methylation groups of the two CpG sites, SII groups, and tumor stage using the χ^2 test. We found a significant consistency between the low SII group and the low methylation group for both CpG sites (figure 5F). However, for cancer stage, we observed a correlation between the methylation group of cg24157392 and cancer stage, while no correlation was found between the methylation group of cg03995631 and cancer stage (figure 5G). The lower SII level in the low methylation group suggests that *BTLA* hypomethylation may be driving immune cell influx, rather than immune cell influx being a consequence of an inflammatory process.

Elevated *DNMT3A/3B* mRNA expression correlated with *BTLA* promoter methylation in melanoma

The underlying mechanisms inducing *BTLA* promoter methylation remain unknown. Recent literature has highlighted the role of DNA methyltransferases, particularly DNMT3A and DNMT3B, in regulating gene expression through DNA methylation. These enzymes are often overexpressed in various cancers, including melanoma,[39] where they play critical roles in tumor progression and immune evasion. To further investigate the potential mechanisms behind *BTLA* promoter methylation, we compared the mRNA expression levels of *DNMT3A* and *DNMT3B* between the high and low methylation groups in 366 metastatic SKCM samples from TCGA. Our results showed that *DNMT3A* and *DNMT3B* were significantly downregulated in the low methylation group (figure 6A). Correlation analysis revealed a significant positive correlation between the mRNA expression levels of *DNMT3A* (R=0.2, p<0.0001) and *DNMT3B* (R=0.16, p=0.0023), and *BTLA* promoter methylation (figure 6B). Furthermore, for 50 melanoma cases randomly selected from our cohort, we conducted quantitative gene expression analysis of *DNMT3A* and *DNMT3B* using qPCR (online supplemental table S4). The expression levels of these genes were then correlated with the methylation status of the *BTLA* promoter. The results revealed a significant positive correlation between *DNMT3B* expression and *BTLA* promoter methylation (R=0.44, p=0.0015). Although no significant correlation was found between *DNMT3A* expression and *BTLA* promoter methylation, a mild correlation was still observed (R=0.25, p=0.086) (figure 6C). These findings suggest that the high expression of DNA methyltransferase genes *DNMT3A* and *DNMT3B* may induce increased *BTLA* promoter methylation, contributing to immune evasion in melanoma, which could explain the shorter survival in the high methylation group.

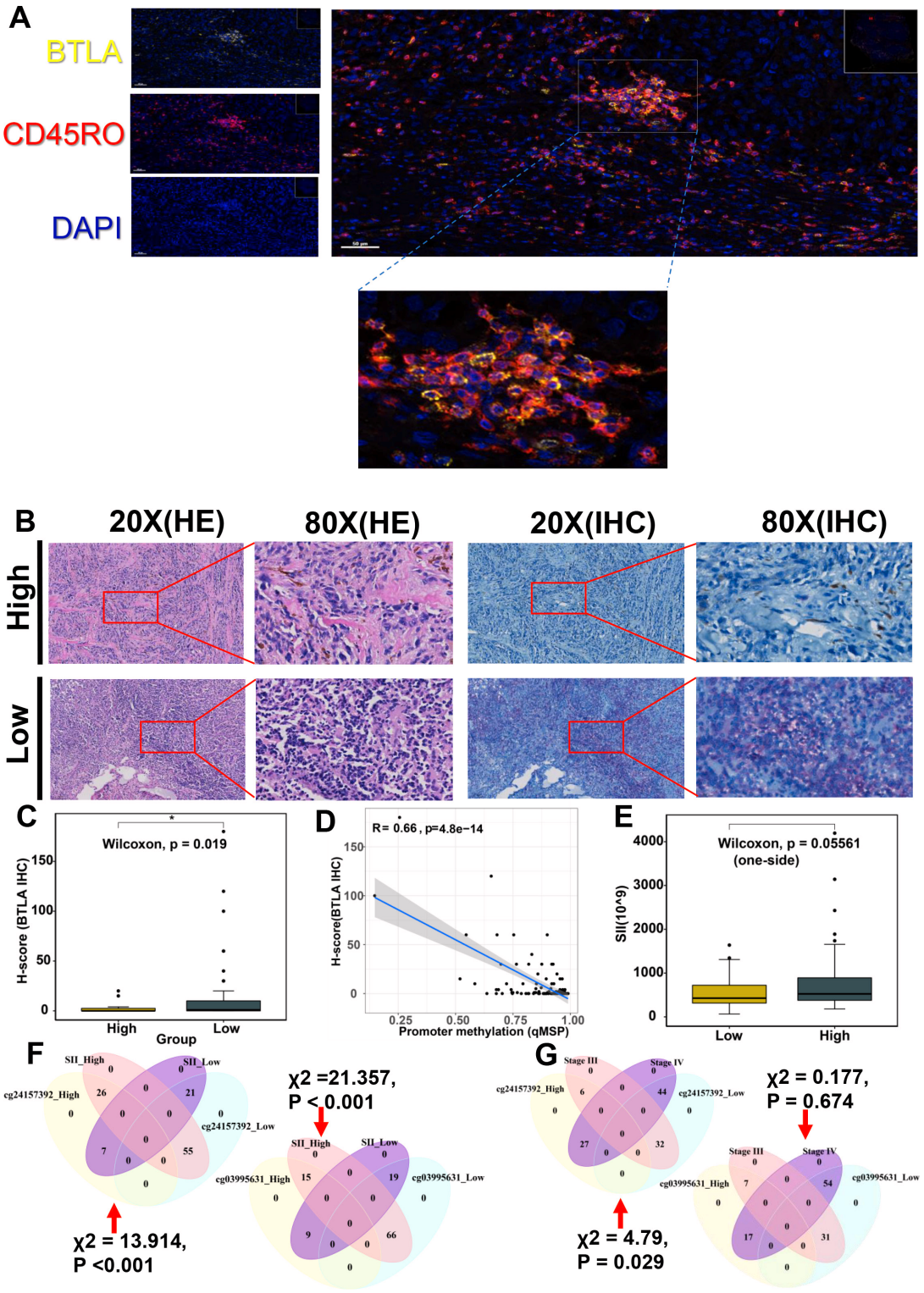

Figure 5 Validation of the negative association of *BTLA* promoter methylation with protein expression and immune cell infiltration. (A) BTLA/CD45RO dual fluorescence immunostaining confirms the colocalization of BTLA (yellow signal) and CD45RO (red signal) within T cells. (B) Representative images show H&E staining of tissue and cell sections and BTLA immunohistochemical (IHC) staining for high and low methylation groups. 20×, 20-fold original magnification; 80×, 80-fold original magnification. (C) Box plot shows the difference of BTLA positive cell fractions between low and high methylation groups. Wilcoxon rank-sum test; *p<0.05. (D) Pearson correlation between promoter methylation and BTLA positive cell fractions by IHC. (E) Box plot shows the difference of systemic immune-inflammation index (SII) between low and high methylation groups. Wilcoxon rank sum one-sided test. (F) The Venn diagram shows the distribution of patients in the high methylation and low methylation groups between the high SII and low SII groups (left panel: cg24157392; right panel: cg03995631). (G) The Venn diagram also illustrates the distribution of patients in the high methylation and low methylation groups across different tumor stages (left panel: cg24157392; right panel: cg03995631).

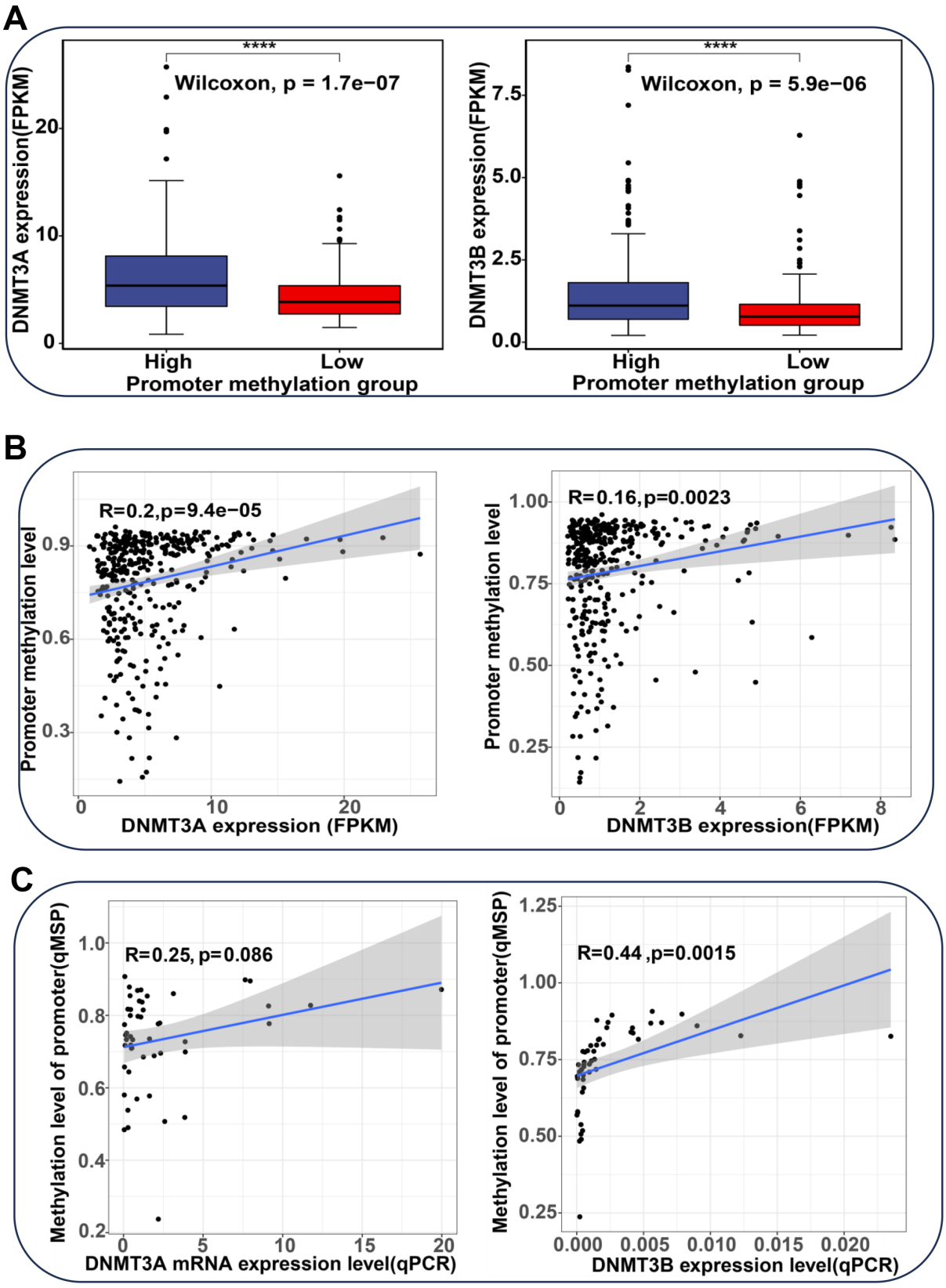

Figure 6 The positive correlations of *BTLA* promoter methylation with *DNMT3A/B* gene expression. (A) Box plots show the gene expression differences of *DNMT3A/B* between high and low promoter methylation groups in 366 TCGA metastatic melanoma. (B) Pearson correlations between *BTLA* promoter methylation level and *DNMT3A/B* gene expression levels in 366 TCGA metastatic melanomas. (C) Pearson correlations between *BTLA* promoter methylation level (qMSP) and *DNMT3A/B* gene expression levels (qPCR) in 50 metastatic melanomas from our FAHZZU cohort. FAHZZU, First Affiliated Hospital of Zhengzhou University; qMSP, quantitative methylation-specific PCR; qPCR, quantitative PCR; TCGA, The Cancer Genome Atlas.All statistical differences of two groups were compared by Wilcoxon rank-sum test; ****p<0.0001.

DISCUSSION

Immune checkpoint inhibitors targeting PD-1/PD-L1 or CTLA-4 have revolutionized the treatment of advanced melanoma, which significantly improved the survival of patients.[2] However, a significant fraction of melanoma patients show resistance to checkpoint inhibitor immunotherapy, and the resistance mechanisms remain poorly understood. BTLA is an inhibitory receptor on immune cells with similar protein structure to CTLA4 and PD1,[3] which plays a critical role in immunosuppression effects via its interaction with HVEM in the TME, leading it to a promising target molecule in immunotherapy.[40] DNA methylation is a stable chemical modification to the DNA, which is involved in epigenetic regulation of gene expression.[31] Understanding the impact of DNA methylation of *BTLA* on its expression and tumor immune microenvironment is urgently needed to help melanoma patients develop reasonable immunotherapy plans.

In our study, we investigated the epigenetic regulation of *BTLA* expression through promoter DNA methylation in melanoma. We assessed the potential of promoter DNA methylation as a biomarker linked to immune cell infiltration, prognosis, and response to immunotherapy. Through comprehensive analysis of public datasets and validation with an in-house cohort, we demonstrated that *BTLA* promoter hypomethylation is closely associated with increased *BTLA* mRNA expression, favorable clinical outcomes, and enhanced immune cell infiltration in the TME. These insights not only advance our understanding of *BTLA's* epigenetic regulation but also propose *BTLA* promoter methylation as a valuable biomarker for predicting OS and response to immunotherapy.

We demonstrated a significant inverse correlation between *BTLA* promoter methylation and its mRNA and protein expression. Specifically, based on the TCGA dataset, we found the hypomethylation of two CpG sites (cg24157392 and cg03995631) in the *BTLA* promoter region was strongly associated with increased mRNA expression in melanoma. Furthermore, we used methylation profile and RNA-seq data to validate these associations in an independent cohort from Newell's study. Finally, we developed the qMSP assay for the quantity of methylation level of cg24157392 and cg03995631 CpG sites and qPCR assay for *BTLA* mRNA expression in melanoma FFPE. Based on qMSP and qPCR experiments, we further validated significant negative correlation between methylation level of cg24157392 and cg03995631 CpG sites and *BTLA* expression in our FAHZZU cohort. IHC analysis for BTLA on 100 melanoma samples from the same FAHZZU cohort also demonstrated *BTLA* promoter hypomethylation is inversely related to BTLA protein expression. This finding aligns with the general understanding of epigenetic regulation, reinforcing that methylation status can directly influence the transcriptional activity of immune checkpoint genes.[13 14] Given BTLA's role as an inhibitory receptor on immune cells, its increased expression might reflect an enhanced regulatory function within the immune system, potentially impacting tumor-immune dynamics.[40] This insight into *BTLA*'s epigenetic regulation adds a new layer of complexity to our understanding of immune checkpoint control and opens avenues for targeted therapeutic interventions.

BTLA expression plays a critical role in the TME of melanoma and influences treatment outcomes.[4] Studies on TILs have revealed that elevated expression levels of *BTLA* are observed in effector CD8+T cells specific to tumor antigens and in memory and effector T cells in melanoma.[5] *BTLA* expression on TILs is variable among patients and influences the effectiveness of immunotherapies such as adoptive cell therapy.[41] In this study, multiplex immunofluorescence staining on three melanoma FFPE tissues from our FAHZZU cohort with BTLA/CD45RO dual fluorescence immunostaining confirmed BTLA is expressed in T lymphocytes. Despite significant findings from previous research on TILs indicating the importance of *BTLA* expression, data from TCGA and our own FFPE melanoma tissue samples show relatively low levels of *BTLA* expression. Our study demonstrates that it is difficult to reliably detect *BTLA* expression using standard qPCR techniques, especially in FFPE samples with significant degradation of RNA along the procedure.[42] But DNA methylation is a stable chemical modification that can be reliably detected using qMSP. Our study demonstrates the strong negative association of DNA promoter methylation with *BTLA* mRNA and protein expression. Then we observed that tumors with promoter hypomethylation exhibited higher infiltration levels of various immune cells, which were estimated by RNA-seq data. Multiplex immunofluorescence, BTLA IHC staining, and HE staining provide strong evidence that *BTLA* promoter hypomethylation is associated with increased immune cell infiltration in melanoma. Our multiplex immunofluorescence analysis, using BTLA and CD45RO dual staining, confirmed that BTLA is expressed on T lymphocytes in melanoma. Furthermore, IHC analysis of 100 melanoma samples revealed that the low methylation group showed an enhanced BTLA protein expression level, and correlation analysis showed a significant negative relationship between *BTLA* promoter methylation levels and BTLA protein expression. Additionally, HE staining images indicated that the low methylation group exhibited higher lymphocyte infiltration, further supporting the link between *BTLA* hypomethylation and enhanced immune cell infiltration. The low methylation group exhibited lower SII levels, which indicates a lower level of systemic inflammation in these patients. This indirectly suggests that the higher immune cell infiltration observed in the low methylation group may be a result of immune cell influx rather than being caused by inflammation. Tumor-infiltrating immune cells, particularly CD8+T cells, B cells, and macrophages, are essential for orchestrating antitumor responses.[34 43 44] The estimated lymphocyte infiltration levels based on pathological images and methylation profiling data also indicated that lower promoter methylation of *BTLA* predicts higher infiltration levels in melanoma.

Our study further elucidated the significant correlations between *BTLA* promoter methylation and immune regulation-related molecules and pathways. Immune checkpoint gene expression regulates immune responses in tumors, offering insights for novel therapies and improving the efficacy and safety of cancer treatments.[45] The inverse correlation between *BTLA* promoter methylation and the expression of other immune checkpoint genes suggests a coordinated regulation of immune inhibitory pathways. This epigenetic interplay could be crucial for maintaining immune homeostasis and preventing autoimmunity while allowing effective antitumor responses. The enrichment of immune-related gene sets and pathways in the tumors with promoter hypomethylation further supports the role of *BTLA* promoter methylation in modulating immune responses. Pathways associated with interferon responses, antigen processing, and presentation were significantly overrepresented, indicating a more active immune milieu in tumors with hypomethylated *BTLA* promoters. These insights provide a deeper understanding of how epigenetic modifications can influence the immune landscape of tumors and impact therapeutic responses.

The high infiltration levels of lymphocytes, including CD8 T cells, B cells, and M1 macrophages, are linked with favorable prognoses and immunotherapy response.[34 43 44] In SKCM patients from TCGA, hypomethylation of CpG sites cg03995631 (p=0.024) and cg24157392 (p=0.0015) in *BTLA* promoter correlated with longer OS, and high *BTLA* mRNA expression was similarly linked to prolonged survival (p<0.0001). These findings were validated in an independent cohort of 50 stage III/IV melanoma tumors, where hypomethylation at these sites also predicted better survival outcomes, with AUCs of 0.88 (cg03995631), 0.94 (cg24157392), and 0.92 (promoter) for 5-year survival prediction. Guo *et al* developed a four-DNA methylation biomarker for predicting OS of patients with cutaneous melanoma. However, in the same validation dataset, the four-DNA methylation biomarker only achieved an AUC of 0.708 for predicting 5-year survival.[36] In contrast, our *BTLA* promoter methylation reached an AUC of over 0.88, with the cg24157392 site specifically achieving an AUC of 0.94. This further demonstrates the effectiveness of *BTLA* promoter methylation in predicting OS in melanoma. In addition, one of the most significant findings of our study is the predictive value of *BTLA* promoter methylation for immunotherapy response. Based on the TCGA dataset, we demonstrated that *BTLA* promoter hypomethylation is significantly associated with enhanced expression levels of previously reported immunotherapy response-predicting biomarkers, including a six-gene IFNγ signature, a related 18-gene IFNγ signature, an effector T cell signature, and a combined IFNγ/effector T cell signature. Then we demonstrated hypomethylation of the cg24157392 and cg03995631 CpG sites was significantly correlated with improved outcomes following immunotherapy in a public dataset and our FAHZZU cohort. By identifying patients with hypomethylation of cg24157392 and cg03995631 CpG sites, clinicians can potentially predict which individuals are more likely to respond favorably to ICB therapy. This stratification could lead to more targeted and effective treatment plans, optimizing therapeutic outcomes and minimizing unnecessary side effects for patients unlikely to benefit from such interventions. The ability to measure *BTLA* promoter methylation accurately through the qMSP assay targeting cg24157392 and cg03995631 CpG sites developed in this study provides a practical approach for integrating this biomarker into clinical practice.

BTLA promoter methylation as a biomarker for predicting immunotherapy response and prognosis has significant advantages over its mRNA expression or protein expression in clinical applications. FFPE tissues are essential in clinical practice, being the backbone of human histopathological diagnoses and molecular testing. Nevertheless, the RNA is prone to degradation during the paraffin embedding process and may further degrade under suboptimal storage conditions. Additionally, chemical modifications induced by formalin fixation can lead to RNA fragmentation and resistance to enzymatic reactions, such as reverse transcription or amplification, making accurate quantification difficult.[46] IHC methods for detecting protein expression, such as PD-L1 IHC scoring to predict immunotherapy response, are widely used in clinical practice. However, IHC scoring faces several challenges, including the temporal and spatial heterogeneity of biomarkers like PD-L1, difficulty in scoring poorly circumscribed or heterogeneous tumors, and preanalytical variables that affect staining quality.[47] Misinterpretation of staining and the exclusion of PD-L1-positive immune cells in certain scoring algorithms further complicate accurate assessment. DNA methylation is a modification at the DNA level that is relatively stable and can be reliably measured in FFPE samples.[48] In this study, we developed the qMSP method to quantify *BTLA* promoter methylation levels in melanoma FFPE samples and validated the accuracy and reproducibility of the qMSP method using targeted methylation sequencing. This method can be readily implemented to stratify patients based on their *BTLA* methylation status, guiding personalized treatment decisions. In addition, previous studies have demonstrated that the use of qMSP to analyze the methylation of circulating cell-free DNA can facilitate the early detection of various cancers.[49] In this study, we employed qMSP to accurately quantify the methylation of the *BTLA* promoter in metastatic melanoma tissue samples. This approach suggests that, for melanoma patients with early metastasis to the lungs or brain, detecting methylation changes in circulating free DNA from blood samples may enable early detection of melanoma metastasis and guide immunotherapy strategies.

Recent literature has highlighted the roles of DNA methyltransferases, particularly DNMT3A and DNMT3B, in the regulation of gene expression through DNA methylation. Both enzymes are often overexpressed in

various cancers, including melanoma,[39 50] where they play significant roles in promoting tumor progression and immune evasion. In addition, studies have shown that overall DNA methylation in melanoma affects PD-L1 expression in melanoma, thereby affecting the ability of melanoma to evade the anti-tumor immune response.[50] In our analysis, we observed a significant positive correlation between the levels of *DNMT3A* and *DNMT3B* and the methylation status of the *BTLA* promoter in melanoma samples. This finding suggests that the upregulation of these methyltransferases may contribute to increased methylation of the *BTLA* promoter, ultimately leading to its suppression. Considering the critical role of BTLA as an immune checkpoint that regulates T-cell activation, its downregulation through methylation could enhance the tumor's ability to escape immune surveillance. Therefore, targeting DNMT3A and DNMT3B may offer a novel therapeutic strategy to restore BTLA expression and improve the efficacy of immunotherapy.

Our study has some limitations. This study first explored the biological and clinical significance of *BTLA* promoter methylation based on TCGA 450K methylation array data. As this chip only includes approximately 450,000 CpG sites, some key sites may not be included. Additionally, in this study, we used a deconvolution statistical method to estimate immune cell infiltration based on RNA-Seq data from TCGA and investigated the impact of *BTLA* promoter methylation on the immune microenvironment. However, this study lacks single-cell level data to investigate the regulation mechanism of the *BTLA* promoter methylation on immune cells, which needs to be further explored in future research. The focus of this study is to determine the potential of *BTLA* promoter methylation as a prognostic biomarker. Nevertheless, it lacks in-depth biological experiments, such as demethylation experiments to explore its impact on the TME. Additionally, due to the limited number of melanoma immunotherapy cases, larger-scale clinical trials to validate the predictive value of *BTLA* promoter methylation are not yet possible. We will continue to collect cases in future studies to further confirm its potential.

CONCLUSIONS

In conclusion, our study establishes *BTLA* promoter hypomethylation as a significant biomarker for favorable prognosis and enhanced response to immunotherapy in melanoma. The correlation between *BTLA* promoter methylation, mRNA and protein expression, immune cell infiltration, and clinical outcomes underscores the importance of epigenetic regulation in shaping tumor-immune interactions. By integrating *BTLA* promoter methylation analysis into clinical practice, we can improve patient stratification and optimize immunotherapy strategies, ultimately advancing the field of precision oncology. Future studies should continue to explore the mechanistic underpinnings of *BTLA* regulation and its broader implications for cancer immunotherapy.

Author affiliations
[1]Department of Pathology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China
[2]The Department of Dermatology, Henan Second Provincial People's Hospital, Zhengzhou, China
[3]Henan Academy of Sciences, Zhengzhou, Henan, China
[4]Department of Oncology, First Affiliated Hospital of Zhengzhou University, Zhengzhou, Hennan, China
[5]Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China
[6]First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan, China

Acknowledgments We acknowledge TCGA and GEO database for providing their platforms and contributors for uploading their meaningful datasets.

Contributors MY provided funding source, designed the study, performed the major data analysis, drafted, edited and reviewed the manuscript; CZhe and YMi collected a part of data, performed wet experiments, and help to generated figures. CY performed a part of wet experiments. LT, CZha, PY and YMa helped to collected a part of data and generate figures. QH provided funding source. PX provided funding source and performed most of wet experiments. SL assisted project management, provided platform support and reviewed and revised the manuscript. WL and GJ designed, oversaw, and supervised the project, and edited, reviewed, and finalized the paper. MY is the guarantor. All authors have read and agreed to the published version of the manuscript.

Funding This work was supported by Henan Province medical science and technology research project: LHGJ20230285 to MY, the National Natural Science Foundation of China (No. 82302961 to QH) and the Youth Innovation Fund of The First Affiliated Hospital of Zhengzhou University (No. 71261 to PX).

Competing interests No, there are no competing interests.

Patient consent for publication Not applicable.

Ethics approval Patient inclusion and sample analyses at FAHZZU were approved by the Institutional Review Board (IRB) of the First Affiliated Hospital of Zhengzhou University (Project ID 2024-KY-0801-002). All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964Declaration of Helsinki and its later amendments.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available in a public, open access repository. Data are available on reasonable request. The partial data supporting the findings of this study are publicly available from TCGA and GEO. The clinical, qMSP and qPCR data of 109 melanoma patients from FAHZZU are available from the corresponding author on reasonable request.

ORCID iD
Minglei Yang http://orcid.org/0000-0001-9957-7608

REFERENCES

1 Morad G, Helmink BA, Sharma P, *et al*. Hallmarks of response, resistance, and toxicity to immune checkpoint blockade. *Cell* 2022;185.
2 Hodi FS, Chiarion-Sileni V, Gonzalez R, *et al*. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced

melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol* 2018;19:1480–92.
3 Watanabe N, Gavrieli M, Sedy JR, *et al*. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat Immunol* 2003;4:670–9.
4 Andrzejczak A, Karabon L. BTLA biology in cancer: from bench discoveries to clinical potentials. *Biomark Res* 2024;12:8.
5 Derré L, Rivals J-P, Jandus C, *et al*. BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination. *J Clin Invest* 2010;120:157–67.
6 Yang M, Lin C, Wang Y, *et al*. Identification of a cytokine-dominated immunosuppressive class in squamous cell lung carcinoma with implications for immunotherapy resistance. *Genome Med* 2022;14:72.
7 Dong X, Song J, Chen B, *et al*. Exploration of the prognostic and immunotherapeutic value of B and T lymphocyte attenuator in skin cutaneous melanoma. *Front Oncol* 2020;10:592811.
8 Patel SP, Kurzrock R. PD-L1 Expression as a predictive biomarker in cancer immunotherapy. *Mol Cancer Ther* 2015;14:847–56.
9 Newell F, Pires da Silva I, Johansson PA, *et al*. Multiomic profiling of checkpoint inhibitor-treated melanoma: Identifying predictors of response and resistance, and markers of biological discordance. *Cancer Cell* 2022;40:88–102.
10 Schübeler D. Function and information content of DNA methylation. *Nature New Biol* 2015;517:321–6.
11 Goltz D, Gevensleben H, Vogt TJ, *et al*. CTLA4 methylation predicts response to anti-PD-1 and anti-CTLA-4 immunotherapy in melanoma patients. *JCI Insight* 2018;3.
12 Hoffmann F, Zarbl R, Niebel D, *et al*. Prognostic and predictive value of PD-L2 DNA methylation and mRNA expression in melanoma. *Clin Epigenetics* 2020;12:94.
13 Niebel D, Fröhlich A, Zarbl R, *et al*. DNA methylation regulates TIGIT expression within the melanoma microenvironment, is prognostic for overall survival, and predicts progression-free survival in patients treated with anti-PD-1 immunotherapy. *Clin Epigenetics* 2022;14:50.
14 Fröhlich A, Sirokay J, Fietz S, *et al*. Molecular, clinicopathological, and immune correlates of LAG3 promoter DNA methylation in melanoma. *EBioMedicine* 2020;59:102962.
15 Available: http://can cergenome.nih.gov/
16 Riaz N, Havel JJ, Makarov V, *et al*. Tumor and microenvironment evolution during immunotherapy with nivolumab. *Cell* 2017;171:934–49.
17 Gide TN, Quek C, Menzies AM, *et al*. Distinct immune cell populations define response to Anti-PD-1 monotherapy and Anti-PD-1/Anti-CTLA-4 combined therapy. *Cancer Cell* 2019;35:238–55.
18 Schwartz LH, Seymour L, Litière S, *et al*. RECIST 1.1 - Standardisation and disease-specific adaptations: perspectives from the RECIST working group. *Eur J Cancer* 2016;62:138–45.
19 Minglei Y, *et al*. BTLA promoter hypomethylation correlates with enhanced immune cell infiltration, favorable prognosis and immunotherapy response in melanoma. 2024. Available: http://decodegene.tech/Supplementary_Table.xlsx
20 Teschendorff AE, Marabita F, Lechner M, *et al*. A beta-mixture quantile normalization method for correcting probe design bias in Illumina Infinium 450 k DNA methylation data. *Bioinformatics* 2013;29:189–96.
21 Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;15:550.
22 Liberzon A, Birger C, Thorvaldsdóttir H, *et al*. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst* 2015;1:417–25.
23 Wu T, Hu E, Xu S, *et al*. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *Innovation (Camb)* 2021;2:100141.
24 Hänzelmann S, Castelo R, Guinney J. GSVA: gene set variation analysis for microarray and RNA-seq data. *BMC Bioinformatics* 2013;14:7.
25 Li T, Fu J, Zeng Z, *et al*. TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res* 2020;48:W509–14.
26 Newman AM, Liu CL, Green MR, *et al*. Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015;12:453–7.
27 Thorsson V, Gibbs DL, Brown SD, *et al*. The Immune Landscape of Cancer. *Immunity* 2018;48:812–30.
28 Saltz J, Gupta R, Hou L, *et al*. Spatial organization and molecular correlation of tumor-infiltrating lymphocytes using deep learning on pathology images. *Cell Rep* 2018;23:181–93.
29 Lehmann U, Kreipe H. Real-time PCR-based assay for quantitative determination of methylation status. *Methods Mol Biol* 2004;287:207–18.
30 McCarty KS Jr, Miller LS, Cox EB, *et al*. Estrogen receptor analyses. Correlation of biochemical and immunohistochemical methods using monoclonal antireceptor antibodies. *Arch Pathol Lab Med* 1985;109:716–21.
31 Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* 2012;13:484–92.
32 Harrison PW, Amode MR, Austine-Orimoloye O, *et al*. Ensembl 2024. *Nucleic Acids Res* 2024;52:D891–9.
33 Bruni D, Angell HK, Galon J. The immune contexture and Immunoscore in cancer prognosis and therapeutic efficacy. *Nat Rev Cancer* 2020;20:662–80.
34 Selitsky SR, Mose LE, Smith CC, *et al*. Prognostic value of B cells in cutaneous melanoma. *Genome Med* 2019;11:36.
35 Rooney MS, Shukla SA, Wu CJ, *et al*. Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015;160:48–61.
36 Guo W, Zhu L, Zhu R, *et al*. A four-DNA methylation biomarker is a superior predictor of survival of patients with cutaneous melanoma. *Elife* 2019;8:e44310.
37 Michie CA, McLean A, Alcock C, *et al*. Lifespan of human lymphocyte subsets defined by CD45 isoforms. *Nature New Biol* 1992;360:264–5.
38 Hu B, Yang X-R, Xu Y, *et al*. Systemic immune-inflammation index predicts prognosis of patients after curative resection for hepatocellular carcinoma. *Clin Cancer Res* 2014;20:6212–22.
39 Micevic G, Muthusamy V, Damsky W, *et al*. DNMT3b Modulates Melanoma Growth by Controlling Levels of mTORC2 Component RICTOR. *Cell Rep* 2016;14:2180–92.
40 Sordo-Bahamonde C, Lorenzo-Herrero S, Granda-Díaz R, *et al*. Beyond the anti-PD-1/PD-L1 era: promising role of the BTLA/HVEM axis as a future target for cancer immunotherapy. *Mol Cancer* 2023;22:142.
41 Radvanyi LG, Bernatchez C, Zhang M, *et al*. Specific lymphocyte subsets predict response to adoptive cell therapy using expanded autologous tumor-infiltrating lymphocytes in metastatic melanoma patients. *Clin Cancer Res* 2012;18:6758–70.
42 Ibusuki M, Fu P, Yamamoto S, *et al*. Establishment of a standardized gene-expression analysis system using formalin-fixed, paraffin-embedded, breast cancer specimens. *Breast Cancer (Auckl)* 2013;20:159–66.
43 Sade-Feldman M, Yizhak K, Bjorgaard SL, *et al*. Defining T Cell States Associated with Response to Checkpoint Immunotherapy in Melanoma. *Cell* 2019;176.
44 Fridman WH, Pagès F, Sautès-Fridman C, *et al*. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012;12:298–306.
45 He X, Xu C. Immune checkpoint signaling and cancer immunotherapy. *Cell Res* 2020;30:660–9.
46 Bai Z, Zhang D, Gao Y, *et al*. Spatially exploring RNA biology in archival formalin-fixed paraffin-embedded tissues. *Cell* 2024;187:6760–79.
47 Humphries MP, McQuaid S, Craig SG, *et al*. Critical Appraisal of Programmed Death Ligand 1 Reflex Diagnostic Testing: Current Standards and Future Opportunities. *J Thorac Oncol* 2019;14:45–53.
48 Papanicolau-Sengos A, Aldape K. DNA Methylation Profiling: An Emerging Paradigm for Cancer Diagnosis. *Annu Rev Pathol* 2022;17:295–321.
49 Li L, Sun Y. Circulating tumor DNA methylation detection as biomarker and its application in tumor liquid biopsy: advances and challenges. *MedComm (2020)* 2024;5:e766.
50 Chatterjee A, Rodger EJ, Ahn A, *et al*. Marked Global DNA Hypomethylation Is Associated with Constitutive PD-L1 Expression in Melanoma. *iScience* 2018;4:312–25.