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Characterization of the Immunological Status of Hypermutated Solid Tumors in the Cancer Genome Analysis Project HOPE

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Abstract. *Background: Many reports demonstrate that a high tumor mutation burden (TMB-H) is closely associated with good prognosis of cancer. However, specific studies investigating the association of various TMB statuses with overall survival in patients with solid tumors are scarce. Patients and Methods: In the present study, we investigated the association of TMB status with overall survival in 5,072 patients with cancer from the HOPE project and clarified the specific mechanism responsible for the good prognosis of the TMB-H group. All tumors were classified into one of four groups based on TMB: ultralow (UL), low (L), intermediate (I) and high (H). Results: The TMB-H group had a better prognosis than the TMB-I and TMB-L groups, but not than the TMB-UL group. Analyzing the expression of 293 immune response-associated genes, 17 genes were up-regulated in the TMB-H group compared to the TMB-I and TNB-L groups, and two genes [CD274 and interferon-γ (IFNG)] were identified as good prognostic factors. Analysis of immune cell populations inside tumors demonstrated that the frequencies of exhausted $CD8^+$ T-cells, activated effector $CD8^+$ T-cells and natural killer cells were significantly higher in the TMB-H group. The T-cell receptor repertoire numbers and the diversity evenness score (DE50) were lower in the TMB-H group than in TMB-UL group; however, no association of the DE50 value with the binding or elution affinity of epitope peptides from neoantigens was found. Conclusion: One possible mechanism for the good prognosis of the TMB-UL group compared to the TMB-H group might be that the TMB-UL group features a balance between immunosuppression and immunostimulation.*

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Key Words: Single nucleotide variant, SNV, tumor mutation burden, TMB, tumor-infiltrating lymphocyte, TIL, T-cell receptor, TCR.

Based on observations of immune checkpoint blockade therapy for various types of solid cancer in a variety of clinical

trials, positive programmed death-ligand 1 (PD-L1) expression and high tumor mutation burden (TMB-H), in addition to high microsatellite instability (MSI-H), are considered possible predictive biomarkers for clinical response and good prognosis (1-5). However, PD-L1 expression is not a definite biomarker in cancer other than some types of solid cancer in which PD-L1 expression is used as a companion biomarker because anti-PD1/PD-L1 blockade has been reported to show obvious antitumor effects in PD-L1-negative cancer (6). On the other hand, TMB-H is accepted as a positive biomarker for antitumor effects and favorable prognosis of patients with MSI-H cancer (7-9).

Many reports demonstrate that TMB-H is closely associated with good prognosis of cancer, particularly in those treated with immune checkpoint blockade (10-13). The association of TMB with survival in terms of single nucleotide variant numbers has not been specifically investigated. Many studies investigating the TMB cutoff value used to determine cancer patient survival have been performed, and approximately 10-20 mutations per megabase are considered possible cutoff values that can be used to determine the prognosis of patients with cancer (14-17). Samstein *et al*. reported that there was an association between higher TMB (more than 10 to 20 mutations per megabase) and improved survival in several types of cancer from an analysis of clinical and genomic data from 1,662 patients with advanced cancer treated with immune checkpoint blockade (17).

Very recently, our group demonstrated that it was possible to classify patients with cancer enrolled in Project HOPE into one of four categories in terms of TMB, namely ultralow (TMB-UL: <1 mutation/megabase), low (TMB-L: 1<mutation/megabase<5), intermediate (TMB-l: 5<mutations/megabase<20) and TMB-H (>20 mutations/megabase), and characterized the specific gene signatures of these four groups (18).

In the present study, based on data from 5,072 patients with cancer enrolled in the HOPE project, we investigated the association of TMB status with survival and clarified the specific mechanism responsible for the good prognosis of the TMB-H group by analyzing various parameters of the immunological tumor microenvironment (TME) including the immune response-associated gene profile and neoantigen and T-cell receptor (TCR) characteristics.

Patients and Methods

Characteristics of patients in project HOPE with TMB-H tumors. More than 5,000 cancer patients were enrolled in the HOPE project from 2014 to 2020 and multiomics analyses including whole-exome sequencing and gene-expression profiling were performed. The TMB was defined as the number of mutations per megabase, which was determined as follows: TMB=N/L, where N and L represent the number of somatic mutations and effective length, respectively. Effective length was defined as the number of base pairs in target regions with a ≥20× depth of coverage in both tumor and matched normal samples. Whole-exome sequencing-based somatic mutations were in this analysis. Single nucleotide variants, indels, synonymous and nonsynonymous mutations were considered in determining the TMB. Based on TMB, all tumors were classified into the TMB-UL, TMB-L, TMB-I, or TMB-H group, as described above. Of the 5,027 enrolled tumor cases, those with hypermutated tumors, *i.e*. with TMB greater than 20 mutations per megabase, were selected (26 cases of stomach cancer and 26 of colorectal cancer) and subjected to human leukocyte antigen (HLA)-DNA typing, neoantigen analysis based on an HLA-binding prediction algorithm, real-time polymerase chain reaction for cytokine and chemokine gene expression, and TCR repertoire profiling. For reference, patients with TMB-UL tumors (26 stomach cancers and 26 colorectal cancers) were also selected as a control group.

Use of the immune response-associated gene panel. The immune response-associated gene panel (293 genes) was described elsewhere (19). In the present study, this panel was utilized for comparative analysis of immune response-associated gene expression among tumor groups with different TMB statuses.

Neoantigen analysis based on an HLA-binding prediction algorithm. Nonsynonymous mutations of hypermutated tumors were collected, and 17 amino acid sequences, including eight forward and eight backward amino acid sequences containing mutated amino acids, were identified from nine sets of 9-mer sequences containing mutated amino acids from each mutated gene. All candidate sequences were analyzed with the HLA-binding prediction algorithm NetMHC4.0 (http: //www.cbs.dtu.dk/services/NetMHC/). All HLA-A locus-restricted sequence candidates with great to moderate binding capacity (<500 nM) or high elution affinity (<2.0%) were considered.

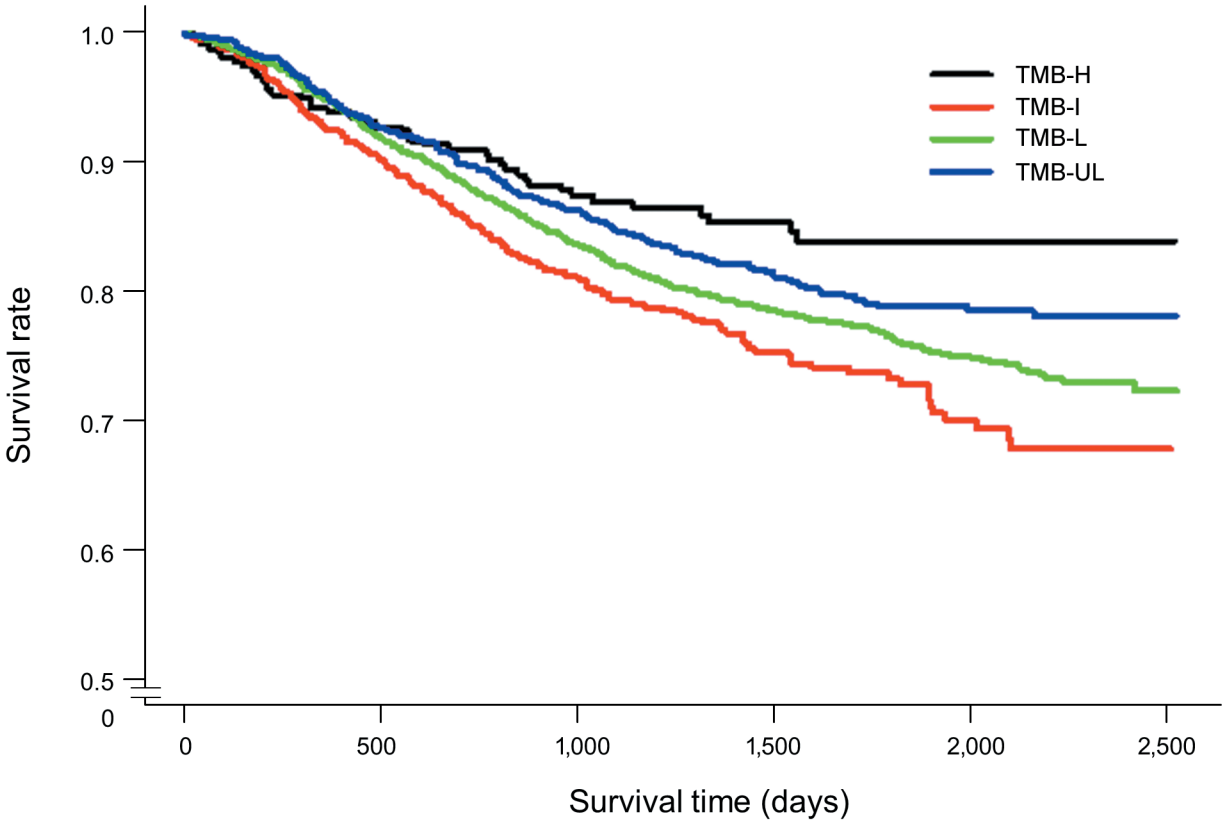
HLA-class I DNA typing of hypermutated tumors. TMB-H tumors were selected (26 of stomach cancer and 26 of colorectal cancer) and used for the HLA-DNA typing. Briefly, DNA was extracted from blood samples using a QIAamp Kit (Qiagen, Hilden, Germany) and subjected to HLA-class I DNA typing using Secore loc a seq kit 25 tests (One Lambda, Inc., West Hills, CA, USA).

TCR gene repertoire analysis of hypermutated tumors using a human TCRα and TCRβ profiling kit. Total RNA was isolated from 19 TMB-H and five TMB-UL tumor tissues from patients with stomach cancer or colorectal cancer and analyzed with the Switching Mechanisms at 5' End of RNA Template (SMARTer™) human TCRα and TCRβ profiling kit (Clontech Laboratories Inc., Mountain View, CA, USA) as described previously (20). TCR repertoire analysis was performed based on hypervariable VDJ segment sequencing using MiTCR, software for TCR sequencing data analysis, which is available from http://mitcr.milaboratory.com/. The diversity evenness score (DE_{50}) was calculated as follows (21):

DE_{50}=Number of rearrangements accounting for 50% of the total map intensity/Total number of rearrangements present

Association of TMB status with immune signaling pathways. Patients in each TMB status group underwent immune signaling pathway profiling based on expression data from a panel of 293 immune response-associated genes by means of ingenuity pathways analysis software (Qiagen).

Statistical analysis. Survival time was compared among the four TMB groups using the Kaplan–Meier method based on the 5-year



	N (%)	TMB (mean)	OS (%)*	HR (95% CI)	*p-Value*
TMB-H	280 (5.6)	68.00±84.56	84.0%	Ref	Ref
TMB-I	609 (12.1)	8.14±3.52	73.3%	1.806 (1.275-2.560)	0.0009
TMB-L	2997 (59.6)	2.83±1.06	76.2%	1.504 (1.093-2.069)	0.0123
TMB-UL	1141 (22.7)	0.46±0.28	78.9%	1.261 (0.899-1.768)	0.1788

Figure 1. *Association of overall survival (OS) with tumor mutation burden (TMB). All 5,027 tumors were classified into four groups based on their TMB: Ultralow (TMB-UL: <1 mutation/megabase), low (1<TMB-L: <5 mutations/megabase), intermediate (5<TMB-I<20 mutations/megabase) and high (TMB-H: >20 mutations/megabase). *The 5-year survival rate among the four TMB groups was compared using the Kaplan–Meier method. CI: Confidence interval; HR: hazard ratio; Ref: reference.*

survival rate. Genes from the 293 immune response-associated gene panel differentially expressed between the TMB-H group and other groups were identified using volcano plot analysis. The association of expression levels of differentially expressed genes (DEGs) with the overall survival time was examined using the Kaplan–Meier method. A comparative analysis of survival time between patients with low expression (less than the median) and patients with high expression (more than the median) of the identified genes in the TMB-H group was performed by the log-rank test using EZR software (22) and Microsoft Excel (Redmond, WA, USA). A heatmap of the expression of DEGs in the different TMB groups was generated using GeneSpring GX software version 13.1.1 (Agilent Technologies, Santa Clara, CA, USA). Values of $p<0.05$ were considered statistically significant.

Results

Association of overall survival time with TMB. The 5,027 pairs of tumors and adjacent normal tissues derived from different cancer types in the HOPE project were analyzed. The numbers of patients with different cancer types were described previously (23). The proportions of patients assigned to the TMB-UL, TMB-L, TMB-I and TMB-H groups were 22.7%, 59.6%, 12.1% and 5.6%, respectively. Analysis of 5-year survival revealed that TMB-H group had a better prognosis than the TMB-I ($p=0.0009$) and TMB-L ($p=0.0123$) groups but did not have a better prognosis than the TMB-UL group

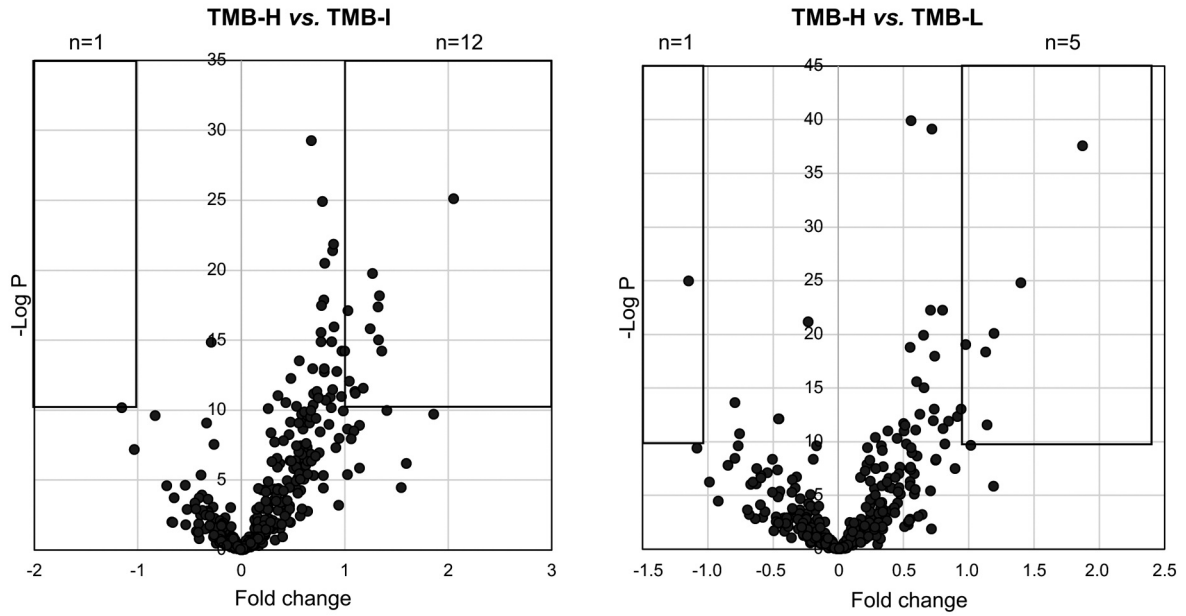


Figure 2. *Identification of immune response-associated genes whose expression was altered in the group with high tumor burden (TMB-H) compared to the groups with intermediate (TMB-I) and low (TMB-L) TMB. A total of 17 immune response-associated genes up-regulated by more than two-fold were identified using volcano plot analysis with Benjamini–Hochberg correction (listed in Table I). The up-regulation of these genes was statistically significant (p<0.05).*

(p=0.1788), with 5-year overall survival rates of 84.0% 73.3%, 76.2% and 78.9, respectively (Figure 1).

The identification of immune response-associated genes with altered expression in the TMB-H group compared with the other groups. Analysis of the expression of the 293-immune response-associated genes in the panel revealed 12 and five unique genes up-regulated in the TMB-H group compared with the TMB-I and TMB-L groups, respectively, and one down-regulated compared with each group (Figure 2 and Table I). In both comparisons, there was a tendency for more genes to be up-regulated in the TMB-H group. Among these 17 DEGs, three were common to both comparisons, namely *CD274,* interferon-γ (*IFNG*) and tumor necrosis factor superfamily member 9 (*TNFSF9*). A volcano plot of the DEGs for the TMB-H *vs*. TMB-UL groups showed 26 genes were up-regulated and 23 down-regulated, but there was no difference in numbers between up-regulated and down-regulated genes (Figure 3 and Table II).

Comparison of the intensity variation in the normalized expression level among TMB groups. The intensity variation in the normalized expression of the 293-immune response-associated genes was compared among the TMB groups. The TMB-H group showed the highest variation; the degree of variation was lower in the TMB-I and TMB-L groups, and the TMB-UL group showed the lowest variation (Figure 4).

Association of the expression of the up-regulated genes with overall survival. The association of 17 genes up-regulated in the TMB H group with overall survival was analyzed by the log-rank test using EZR software and the median expression value. Ultimately, high expression of two genes, *CD274* and *IFNG*, was found to be significantly associated with better overall survival, with 5-year rates of 75.5% *vs*. 79.3% for those with low *CD274* expression (p=0.0024), and 75.2% *vs*. 79.5% for those with low *IFNG* expression; *TNFSF9* gene expression was not associated with prognosis (p=0.687), (data not shown).

Characterization of neoantigen candidates and TCR repertoire profiling in patients with TMB-H stomach and colorectal cancer. Sufficient material was available from 38 TMB-H tumors (19 stomach cancer and 19 colorectal cancer) and 10 TMB-UL tumors (five stomach cancer and five colorectal cancer) for investigation of HLA-DNA type, single nucleotide variant number, HLA-A-matched neoantigen candidate number, TCR repertoire characteristics and DE50 score (data not shown). There was a significant difference in TCR repertoire characteristics between the TMB-H and TMB-UL groups in colorectal cancer but not stomach cancer (Figure 5A). Specifically, the TCRβ repertoire number was significantly higher in the TMB-UL group. Similarly, analysis of the DE50 score showed a lower number in the TMB-H group than the TMB-UL group in colorectal cancer for both TCRα and TCRβ, but the difference was not significant (Figure 5B and C).

Table I. *Genes with altered expression in the group with high tumor mutation burden (TMB-H) compared to the groups with intermediate (TMB-I) and low (TMB-L) tumor mutation burden.*

Altered in TMB-H *vs.*	Probe name	Log FC	–LogP	Gene symbol	Encoded protein
TMB-I	A_23_P207564	1.00	14.2	*CCL4*	CCL4
	A_33_P3326588	1.03	17.1	*TNFRSF10D*	TNFRSF10D (TRAILR4)
	A_33_P3316273	1.04	12.1	*CCL3*	CCL3
	A_23_P117602	1.10	11.3	*GZMB*	Granzyme B
	A_23_P256724	1.10	11.2	*TNFRSF10C*	TNFRSF10C (TRAILR3)
	A_21_P0000178	1.18	11.6	*TREM1*	TREM1
	A_23_P338479	1.25	15.8	*CD274*	CD274 (PD-L1)
	A_23_P304356	1.27	19.8	*CLEC5A*	CLEC5A (MDL-1)
	A_23_P392942	1.32	17.4	*MSR1*	MSR1 (CD204)
	A_23_P151294	1.33	15.0	*IFNG*	Interferon-γ
	A_23_P200728	1.33	18.2	*CD16*	CD16 (FcγRIII)
	A_33_P3319905	1.36	14.2	*TREM1*	TREM1
	A_33_P3397763	2.05	25.1	*TNFSF9*	TNFSF9 (CD137L)
	A_23_P389897	–1.15	10.2	*NGFR*	NGFR (CD271)
TMB-L	A_23_P338479	1.13	18.4	*CD274*	CD274 (PD-L1)
	A_23_P18452	1.14	11.6	*CXCL9*	CXCL9 (MIG)
	A_23_P145485	1.19	20.1	*ULBP2*	ULBP2 (NKG2DL2)
	A_23_P151294	1.4	24.8	*IFNG*	Interferon-γ
	A_33_P3397763	1.87	37.6	*TNFSF9*	TNFSF9 (CD137L)
	A_23_P259442	–1.15	25	*CPE*	CPE

FC: Fold change.

Association of the TCR repertoire profile with TMB status and neoantigen candidate peptide binding affinity. All HLA-A locus-restricted sequence candidates with potent binding capacity (<500 nM) or elution affinity (<2.0%) were selected for analysis. No significant association of DE50 scores for TCRα and TCRβ with the binding or elution affinity of epitope peptides was observed for colorectal or stomach cancer (Figure 6).

Association of immune cell populations with TMB status. The proportions of patients with exhausted $CD8^+$ T-cells ($PD1^+TIM3^+$), activated effector $CD8^+$ T-cells ($PD1^+IFNG^+$ or $PD1^+GZMB^+$) and mature dendritic cells ($CD11c^+CD83^+HLA\text{-}DR^+$) were significantly higher in the TMB-H group than in the other groups (Table III). The frequency of patients with regulatory T-cells ($CD25^+FOXP3^+$) tended to be reduced in the TMB-H group but this was not significant. Additionally, the frequency of those with activated natural killer (NK) cells ($CD16^+NCR1^+$) was significantly increased in the TMB-H group. Interestingly, the frequency of patients with mature B-cells ($CD19^+CD20^+HLA\text{-}DR^+$) was significantly higher in the TMB-H group than in the TMB-I and TMB-L groups but not in the TMB-UL group.

Association of TMB status with immune signaling pathways. Immune pathway-specific characterization of each TMB group was performed using ingenuity pathway analysis software to generate a radar chart. The Z score of the TMB-

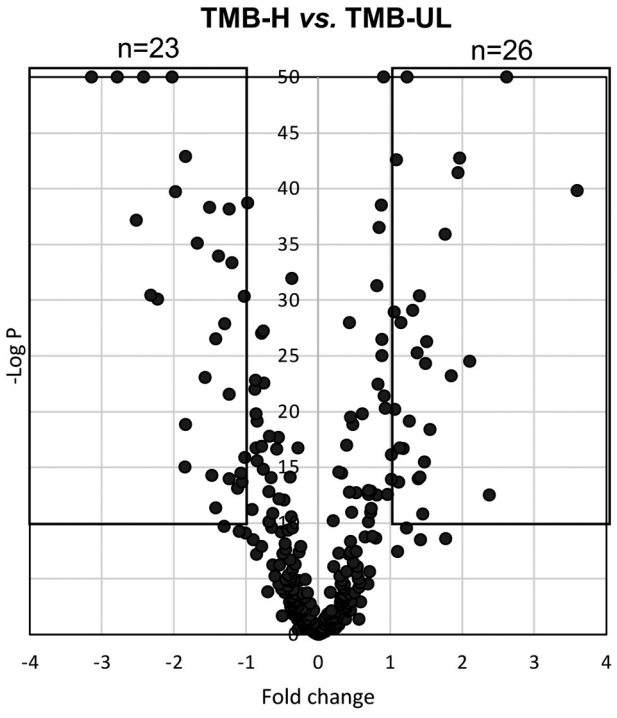


Figure 3. *Identification of immune response-associated genes whose expression was altered in the group with high tumor mutation burden (TMB-H) compared to the group with ultralow TMB (TMB-UL). Immune response-associated genes whose expression was altered by more than two-fold were identified using volcano plot analysis with Benjamini–Hochberg correction. A total of 26 were up-regulated and 23 were down-regulated, as listed in Table II.*

Table II. *Immune response-associated genes whose expression was altered in the group with high tumor mutation burden (TMB-H) compared to group with ultralow burden (TMB-UL).*

	Probe name	Fold change	Gene symbol	Encoded protein
Up-regulated (26 genes)	A_23_P304356	1.02	*CLEC5A*	CLEC5A (MDL-1)
	A_23_P207564	1.02	*CCL4*	CCL4
	A_24_P12401	1.06	*VEGFA*	VEGF-A
	A_23_P76078	1.06	*IL23A*	IL23A
	A_23_P255653	1.08	*TNFRSF10A*	TNFRSF10A (TRAILR1)
	A_21_P0000178	1.11	*TREM1*	TREM1
	A_33_P3316273	1.13	*CCL3*	CCL3
	A_33_P3374210	1.15	*MKI67*	KI67
	A_23_P112026	1.17	*IDO1*	IDO-1
	A_23_P200728	1.26	*CD16*	CD16 (FcγRIII)
	A_24_P218979	1.31	*CDCA3*	CDCA3
	A_23_P49338	1.37	*TNFRSF12A*	TNFRSF12A (TWEAKR)
	A_33_P3214550	1.39	*CXCR2*	CXCR2
	A_23_P218646	1.4	*TNFRSF6B*	TNFRSF6B (Dcr3)
	A_24_P303091	1.41	*CXCL10*	CXCL10
	A_23_P17065	1.45	*CCL20*	CCL20
	A_33_P3319905	1.48	*TREM1*	TREM1
	A_23_P338479	1.5	*CD274*	CD274 (PD-L1)
	A_23_P18452	1.55	*CXCL9*	CXCL9
	A_23_P117602	1.76	*GZMB*	Granzyme B
	A_23_P133408	1.84	*CSF2*	CSF2
	A_23_P151294	1.94	*IFNG*	Interferon-γ
	A_23_P145485	1.96	*ULBP2*	ULBP2 (NKG2DL2)
	A_23_P49155	2.1	*CDH3*	CDH3 (P-cadherin)
	A_23_P501754	2.37	*CSF3*	CSF3
	A_32_P87013	3.59	*CXCL8*	CXCL8
Down-regulated (23 genes)	A_23_P10121	–2.53	*SFRP1*	SFRP1
	A_23_P123853	–2.33	*CCL19*	CCL19
	A_33_P3363799	–1.98	*NCAM1*	NCAM1 (CD56)
	A_23_P37736	–1.85	*TNFRSF17*	TNFRSF17 (BCMA)
	A_24_P226755	–1.85	*TOX*	TOX
	A_33_P3406567	–1.84	*CD20*	CD20
	A_33_P3413468	–1.68	*EDA2R*	EDA2R (TNFRSF27)
	A_33_P3383970	–1.57	*TLR10*	TLR10
	A_23_P70719	–1.51	*LAMA2*	LAMA2
	A_23_P113572	–1.48	*CD19*	CD19
	A_23_P368805	–1.42	*B7H7*	HHLA2
	A_33_P3250680	–1.42	*CD40LG*	CD40 ligand (CD154)
	A_23_P85240	–1.38	*TLR7*	TLR7
	A_24_P412156	–1.3	*CXCL12*	CXCL12
	A_23_P84705	–1.24	*TNFRSF13B*	TNFRSF13B (TACI, CD267)
	A_23_P352266	–1.24	*BCL2*	BCL-2
	A_23_P9402	–1.24	*CNTFR*	CNTFR
	A_33_P3218975	–1.2	*ENTPD1*	NTPDase1
	A_23_P138706	–1.12	*ADRA2A*	ADRA2A
	A_23_P48088	–1.08	*CD27*	CD27
	A_33_P3358923	–1.06	*BTLA*	BTLA (CD272)
	A_33_P3221303	–1.03	*CCR10*	CCR10
	A_24_P231104	–1.02	*LEPR*	Leptin receptor (LEPR)

H group was set at 0 and compared to that of other TMB groups in terms of various immune response-associated signaling pathways. The TMB-I group showed significant inhibition of T-helper (Th) responses, inflammatory signals, cytokine/chemokine signals, interleukin 17 signaling, dendritic and NK cell pathways, and B-cell responses compared to the TMB-H group (Figure 7). The TMB-L group exhibited moderate down-regulation of inflammatory

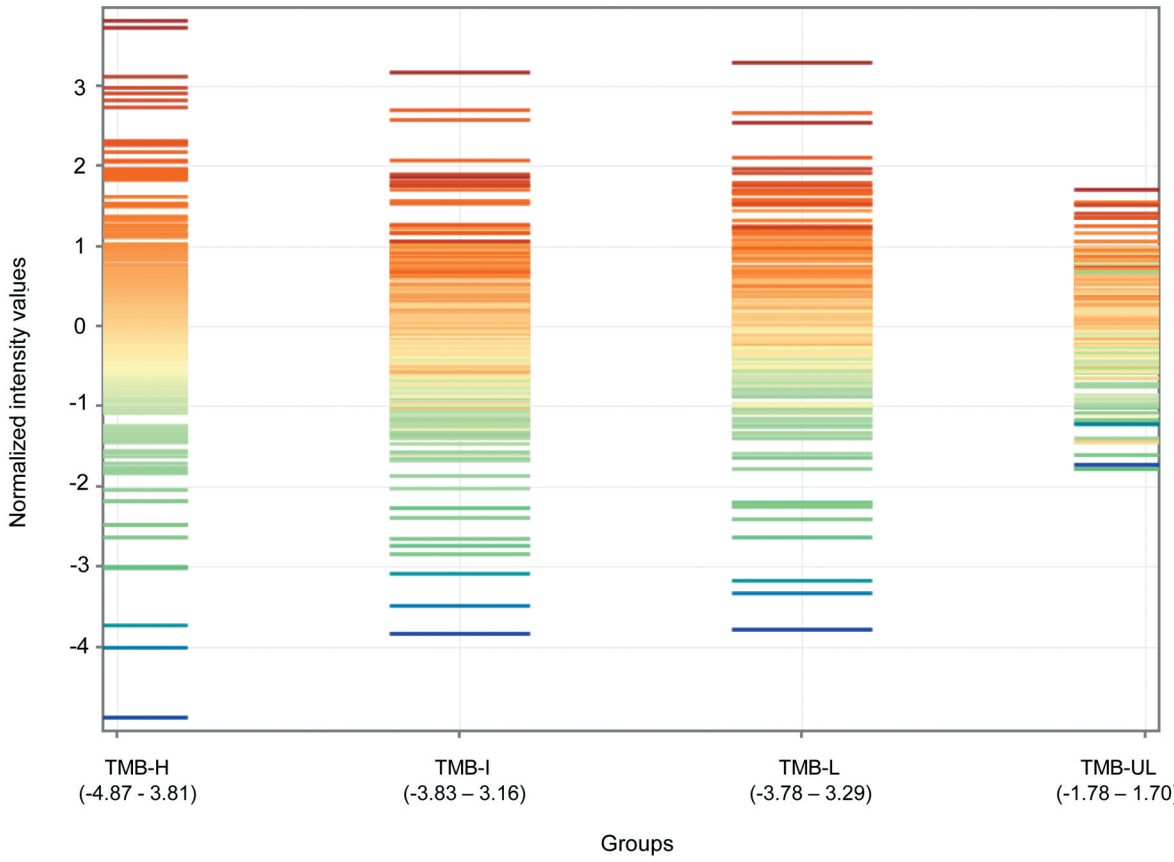


Figure 4. *Comparison of the intensity variation in normalized expression levels of immune response-associated genes for groups with high (TMB-H), intermediate (TMB-I) and low (TMB-L) tumor burden. The red and blue colors in the panel reflect the relative expression level of individual genes, as indicated in the scale bar (log2-transformed scale).*

signaling and the dendritic-NK cell pathway. Interestingly, the TMB-UL group had a similar profile to the TMB-H group; but only the PD1/PD-L1 signaling pathway was inhibited in the TMB-UL group compared to the other groups. For reference, the calculated Z scores for each TMB group were as follows: TMB-H 0, TMB-I –25, TMB-L –4.5 and TMB-UL –1.5.

Discussion

Based on the results of anti-immune checkpoint antibody-based clinical trials, positive PD-L1 expression, TMB-H and MSI-H status are thought to be possible prognostic factors for cancer patients treated with PD-1/PD-L1 blockade (1-5). In addition, tumor-infiltrating lymphocytes (TILs) or $CD8^+$ T-cells, as factors of the TME, can also be important parameters for predicting the prognosis of patients with cancer (24-26).

In the current study, we focused on hypermutated tumors registered in the HOPE project and investigated the association of TMB status with overall survival and various other parameters, such as expression of specific immune-response genes, specific immune cell populations, TCR repertoire profile and signaling pathway statuses.

Firstly, the association of TMB status with overall survival was determined using survival data of 5,027 patients with solid cancer, and those with TMB-H tumors had a significantly better overall survival rate than those with TMB-L and TMB-I tumors but not than those with TMB-UL tumors. A few precise and specific survival analyses of various TMB groups have been reported (14-17), but our comparison of the TMB-H with the TMB-UL group in terms of overall survival is a novel observation. The specific mechanism responsible for the TMB-H group not having better survival compared to the TMB-UL group is thought to be as follows: i) The comparison of TMB-H and TMB-UL by volcano plot analysis showed 26 up-regulated genes and 23 down-regulated genes, but there was no difference between the up-regulated and down-regulated genes. Thus, there was a balance between the expression of antitumor

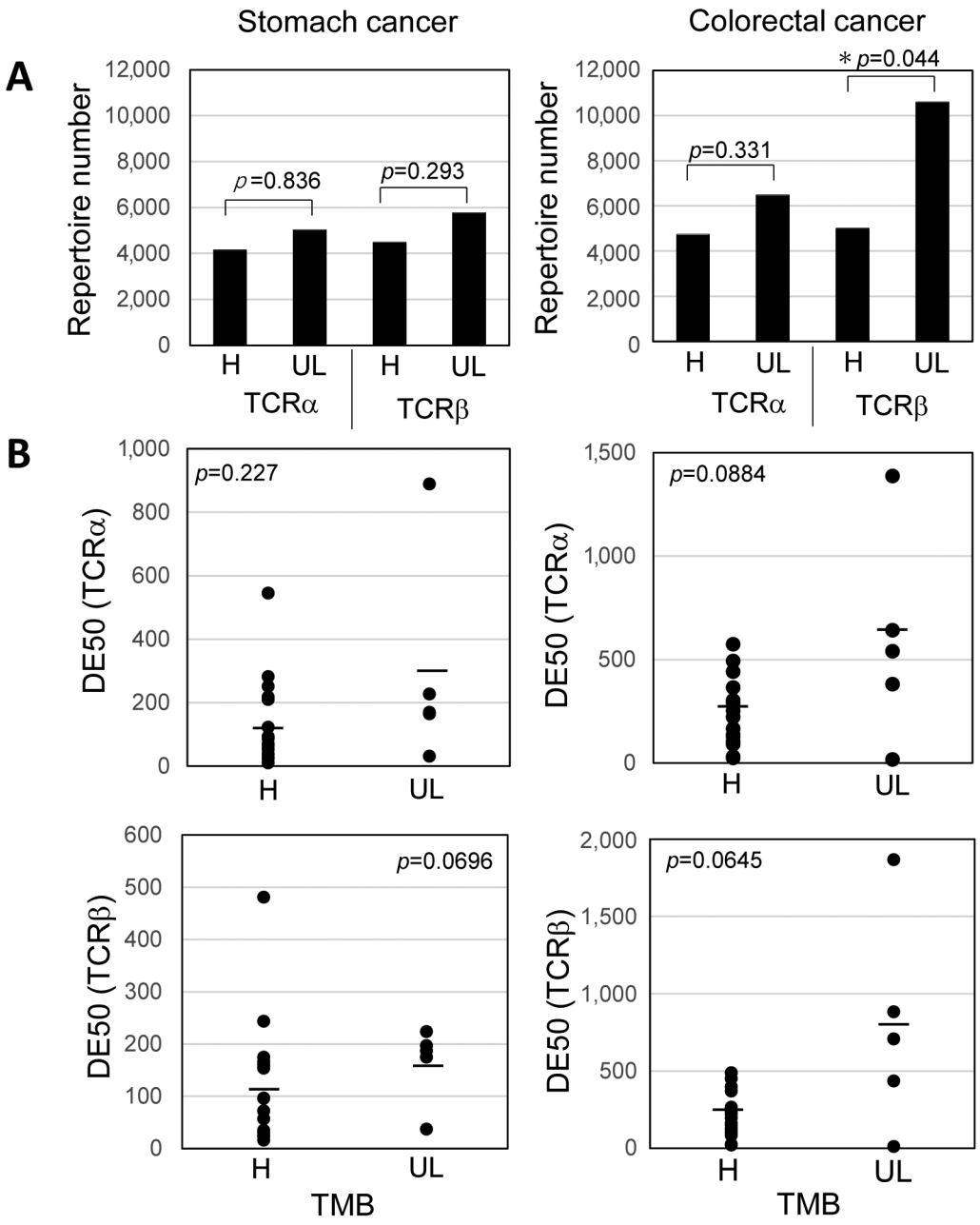


Figure 5. *T-Cell receptor (TCR) repertoire characteristics according to tumor burden (TMB) in patients with stomach and colorectal cancer. TCR repertoire number (A) and diversity evenness 50 (DE50) scores (B) were determined for patients with stomach and colorectal cancer with high (H) and ultralow (UL) TMB using a TCR repertoire profiling kit. Statistical analysis of the differences between the TMB-H and TMB-UL groups in terms of repertoire level and DE50 score was performed using the Mann–Whitney U-test. *Significantly different.*

genes and immune-suppressive genes in the TMB-UL group; ii) Immune signaling pathway analysis indicated that the groups had similar pathway profiles except for the PD1/PD-L1 immunotherapy pathway, which might suggest that the TMB-UL group features a balance of immune-stimulation and immune-suppression in the TME.

Secondly, comparison of the expression of 293 immune response-associated genes among the TMB status groups was successfully performed. CD274 (PD-L1) and IFNG were found to be genes positively associated with prognosis in the TMB-H group compared to the TMB-L and TMB-I groups, and these genes contribute to the Th1 antitumor response

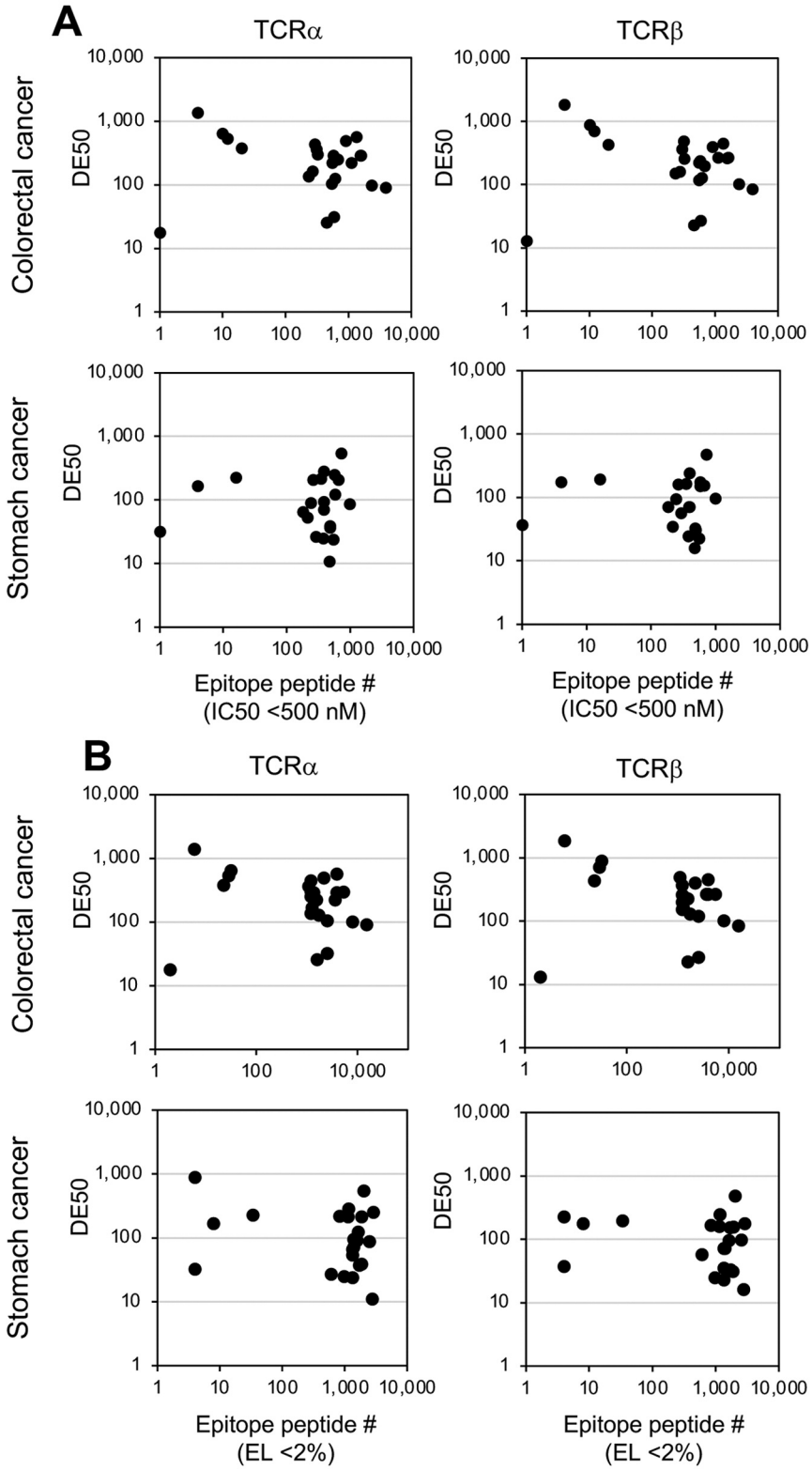


Figure 6. *T-Cell receptor (TCR) repertoire profile according to neoantigen peptide-binding affinity. The association of TCRα and TCRβ diversity evenness 50 (DE50) scores with the binding (A) and elution (EL) affinity (B) of neoantigen epitope peptides was investigated in patients with colorectal and stomach cancer. Note that axes are logarithmic. The association of DE50 scores for TCRα and TCRβ with neoantigen candidate numbers with binding affinity was assessed using Pearson's correlation coefficient. IC50: Half-maximal inhibitory concentration.*

Table III. *Comparison of specific cell populations among groups with different tumor mutation burden (TMB).*

Cell population	Group, n (%)					*p*-Value
	All	TMB-H	TMB-I	TMB-L	TMB-UL	
Total cases, n	4651	263	574	2807	1007	
$CD3^+CD8^+$	662 (14.2)	41 (15.6%)	63 (11.0)	374 (13.3)	184 (18.3)	<0.01 *vs*. TMB-I and -L
$CD3^+CD8^+PD1^+$	722 (15.5)	56 (21.3%)	79 (13.8)	395 (14.1)	192 (19.1)	<0.01 *vs*. TMB-I and -L
$CD3^+CD8^+PD1+IFNG^+$	580 (12.5)	55 (20.9%)	68 (11.9)	331 (11.8)	126 (12.5)	<0.01 *vs*. all others
$CD3^+CD8^+PD1^+GZMB^+$	578 (12.4)	52 (19.8%)	70 (12.2)	335 (11.9)	121 (12.0)	<0.01 *vs*. all others
$CD3^+CD8^+PD1^+TIM3^+$	423 (9.09)	43 (16.4%)	43 (7.49)	221 (7.87)	116 (11.5)	<0.01 *vs*. all others
$CD3^+CD8^+PD1^+TNFSF9^+$	408 (8.77)	44 (16.7%)	36 (6.27)	233 (8.30)	95 (9.43)	<0.01 *vs*. all others
$CD25^+FOXP3^+$	40 (0.86)	2 (0.76%)	5 (0.87)	17 (0.61)	16 (1.59)	0.074
$CD19^+CD20^+HLA\text{-}DR^+$	507 (10.9)	35 (13.3%)	36 (6.27)	271 (9.65)	165 (16.4)	<0.01 *vs*. TMB-I and -L
$CD19^+CD27^+CD138^+$	727 (15.6)	31 (11.8%)	79 (13.8)	407 (14.5)	210 (20.9)	0.147
$CD11c^+CD83^+HLA\text{-}DR^+$	468 (10.1)	41 (15.6%)	37 (6.45)	247 (8.80)	143 (14.2)	<0.01 *vs*. TMB-I and -L
$CD14^+CD11b^+ARG1^+$	74 (1.59)	9 (3.42%)	5 (0.87)	34 (1.21)	26 (2.58)	0.203
$CD16^+NCR1^+$	352 (7.57)	43 (16.4%)	32 (5.57)	186 (6.63)	91 (9.04)	<0.01 *vs*. all others
$CSFR1^+MSR1^+$	370 (7.96)	16 (6.08%)	17 (2.96)	180 (6.41)	157 (15.6)	<0.01 *vs*. UL
$CSFR1^+CD68^+$	304 (6.54)	15 (5.70%)	14 (2.44)	137 (4.88)	138 (13.7)	<0.01 *vs*. UL

TMH-H: High TMB; TMB-I: intermediate TMB; TMB-L: low TMB; TMB-UL: ultra-low TMB. The ratio of the intensity of expression between the tumor tissue and surrounding normal tissue was calculated from normalized values. A ratio of >2.0 was considered positive. The frequency of each immune cell population was compared using Pearson's chi square test. Values of $p<0.05$ were considered significant.

activating effector $CD8^+$ T-cells. In a previous study, we reported a 4-immune type classification of solid tumors based on PD-L1 and CD8B gene expression, and the type A ($PD\text{-}L1^+CD8B^+$) group was found to have a good prognosis (19). Interestingly, the TMB-H group and $PD\text{-}L1^+CD8B^+$ group showed a similar immunological signature, such as activated effector T-cell markers, which suggests that the TME is immune-activated ('hot') in the TMB-H group. In addition, we investigated the various immune cell populations in the TMB-H group. Interestingly, the proportion of patients with mature B-cells ($CD19^+CD20^+HLA\text{-}DR^+$) was higher in the TMB-H group than in the TMB-I and TMB-L groups. The presence of the B-cell population in TILs been identified as a good prognostic factor in several studies (27, 28); therefore, the presence of mature B-cells in the TME might contribute to the good prognosis of patients with TMB-H tumors.

Thirdly, TCR repertoire profiling revealed that the TMB-H group had a smaller TCRβ repertoire and DE50 value, particularly in patients with colorectal cancer. However, the TCR DE50 value did not show any association with neoantigen epitope number or TIL number, probably because of the small number of tumor cases analyzed in the TCR profiling. These results may suggest that TCR DE50 might not be a universal biomarker of immunological status and prognosis for patients with hypermutated tumors. Generally, it is accepted that when appropriate tumor-specific neoantigens are recognized by the T-cell immune system, specific TCR repertoire variation and DE50 values should decline, resulting in a durable antitumor response (29-34). Hogan *et al*. demonstrated that low ED50 values were predictive of a longer progression-free survival and good response to PD1 blockade prior to treatment by immune checkpoint blockade (21). In the near future, more TMB-H and TMB-L tumor cases and more reliable TCR repertoire data relevant to tumor-specific neoantigens are needed.

Finally, based on the results from the present study comparing TMB with other parameters, TME biomarkers in hypermutated tumors were evaluated. We previously reported that TMB and PD-L1 expression were strongly positively correlated (35). We also found this in the present study, suggesting that TMB-H tumors tend to express high levels of the PD-L1 gene. TIL status showed a trend towards being associated with increased accumulation of activated effector T-cells in the TMB-H group. Additionally, TMB was found to be a potent prognostic biomarker in the overall survival analysis. Interestingly, there was no survival benefit for the TMB-H group compared to TMB-UL group. Black *et al*. demonstrated that lower genetic instability is associated with a better prognosis (15). These results might suggest that the TMB-UL group features a balance between immunosuppression and immunostimulation, which might result in a better prognosis. We plan to further investigate the precise mechanism responsible for these observations in the future.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

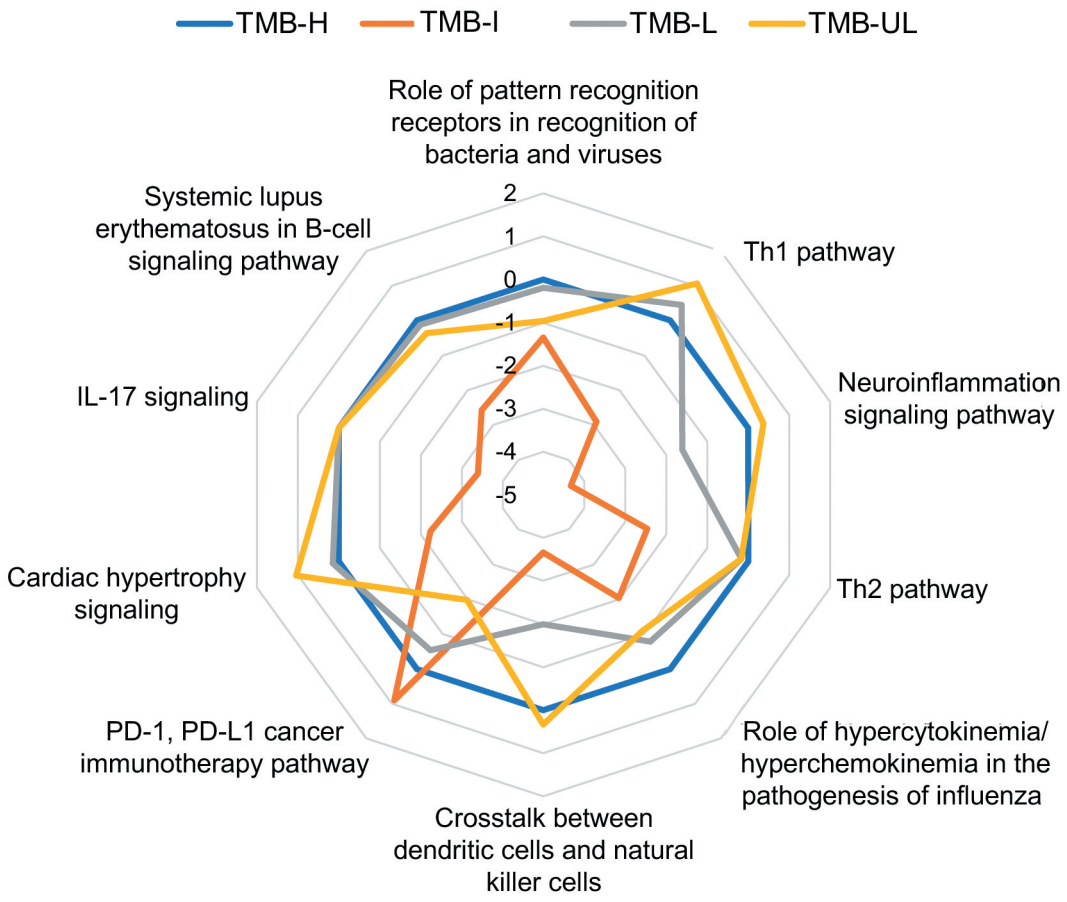


Figure 7. *Association of tumor burden (TMB) status with immune signaling pathways. The 5,027 patients with cancer with high (TMB-H), intermediate (TMB-I), low (TMB-L) and ultralow (TMB-UL) tumor mutation burden underwent profiling of 10 immune signaling pathways based on the expression of immune response-associated genes by means of ingenuity pathway analysis (IPA) software (Qiagen). The Z score of the TMB-H group was set at 0 and compared to that of other TMB groups in terms of the status of 10 immune response-associated signaling pathways. Th1/Th2: T-Helper type 1/type 2; IL-17: interleukin 17.*

Authors' Contributions

YA participated in the design of the study and drafting of the article and was responsible for completing the study. AI, HM, CM, AK and TA performed immunological *in vitro* assays. TN, RK, AN and KM were responsible for the statistical analysis. AS, YO, MT, KU, YH, YK, HK and Mitsuru Takahashi participated in collecting clinical samples. Kenichi Urakami, YS and KO were responsible for genetic analysis of clinical specimens. TS was responsible for preparing specimens for TIL analysis. Hirotsugu Kenmotsu and KY reviewed the article. All Authors read and approved the final draft.

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