Immune response-associated gene profiling in Japanese melanoma patients using multi-omics analysis

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Abstract. Project High-tech Omics-based Patient Evaluation (HOPE), including comprehensive whole-exome sequencing (WES) and gene expression profiling (GEP) using freshly resected tumor specimens, has been in progress since its implementation in 2014. Among a total of 1,685 cancer patients, 13 melanoma patients were registered in the HOPE Project and were characterized using multi-omics analyses. Among the 13 melanoma patients, 4 were deceased, and 9 were alive. The mean overall survival (OS) and relapse-free survival (RFS) times of the melanoma patients were 16.9 and 14.7 months, respectively. Previously, we developed an immune response-associated gene list, which consisted of 164 genes in Project HOPE, for evaluating the immunological status. In the present study, the association of immune response-associated gene expression with immunological parameters, such as programmed death-ligand 1 (PD-L1) and CD8 expression levels, single nucleotide variant (SNV) number, and Vogelstein driver gene mutation number, was investigated. With respect to PD-L1 expression, both immuno-suppression and immuno-stimulation-related genes were upregulated in PD-L1-positive melanomas. In contrast, regarding Vogelstein driver mutations, several T-cell activation-related genes were significantly downregulated in the high driver gene mutation group. In addition, many T-cell activation-related genes were upregulated in the CD8-positive melanomas. The correlation of immune response-associated gene expression with the survival time of the melanoma patients was investigated. Eight specific genes were commonly identified as genes that were significantly correlated for both the overall OS and RFS time, which could be possible prognostic factors for melanoma patients. These results revealed that an immune response-associated gene panel could be an informative tool for evaluating the immunological status prior to clinical immunotherapy in the upcoming era of genomic cancer medicine.

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Abbreviations: WES, whole-exome sequencing; GEP, gene expression profiling; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; NGS, next-generation sequencing; SNV, single nucleotide variant; TIL, tumor-infiltrating lymphocyte; OS, overall survival; RFS, relapse-free survival

Key words: Japanese melanoma, whole-exome sequencing, gene expression profiling, overall survival, relapse-free survival

Introduction

Programmed death-ligand 1 (PD-L1) and PD-1 expression is variably regulated in immune cells and tumor cells to maintain immunological tolerance, which controls the occurrence of an autoimmune reaction against self-antigens (1,2). PD-L1-expressing antigen-presenting cells, such as monocytes, macrophages, dendritic and tumor cells regulate excess immune reactions and inhibit activated T-cell function (3,4). Meanwhile, PD-1, the receptor for PD-L1, is expressed on activated T, B and NK cells in the tumor microenvironment. Anti-PD-1 blockade therapy promotes exhaustive marker-positive T-cell expansion and survival (5), resulting in an antitumor response *in vivo*.

Since the recent success of immune checkpoint antibodies, such as ipilimumab and nivolumab, as reported for metastatic melanoma patients, many ongoing clinical trials have been underway to evaluate their efficacy in various solid cancers other than melanomas (6-8). Despite these promising results, the response rate associated with the single antibody treatment is ~20-40% while 60-70% of cancer patients belong to the non-responding group. Furthermore, it is still difficult to accurately predict the responders to antibody therapy based on the current preclinical studies (9,10).

In the present study, we used a previously reported immune response-associated gene panel consisting of 164 genes (56 antigen-presenting cells and T-cell-associated genes,

34 cytokine- and metabolism-associated genes, 47 TNF and TNF receptor superfamily genes and 27 regulatory T-cell-associated genes) (11). The present study investigated the association of the gene panel expression with immunological and clinical parameters, such as i) PD-L1 expression; ii) a high mutation load [single nucleotide variant (SNV) number]; iii) a driver gene mutation; iv) CD8 expression; and v) survival time, using the genomic data from 13 melanoma patients in the Project High-tech Omics-based Patient Evaluation (HOPE). Since 2014, 1,685 cancer patients have been enrolled in Project HOPE in which the simultaneous analyses of whole-exome sequencing (WES) and gene expression profiling (GEP) have been performed (12,13). We aimed to evaluate the immunological status in the tumor tissues using next-generation sequencing and to better obtain a prediction of the responders to immune checkpoint antibody treatment through suitable biomarker detection.

Materials and methods

Patient registration. Project HOPE uses comprehensive whole-exome sequencing and gene expression profiling of various tumor tissues and is conducted in accordance with the 'Ethical Guidelines for Human Genome and Genetic Analysis Research' in Japan. Informed consent was obtained from all the patients participating in Project HOPE, and the study was approved by the Institutional Review Board of Shizuoka Cancer Center (SCC), Japan. Tumor tissues, along with the surrounding normal tissues, were dissected from surgical specimens by trained pathologists. A total of 1,685 cancer patients were registered in Project HOPE from 2014 to 2015. Characteristics of the 13 melanoma patients listed are shown in Table I.

Comprehensive gene expression analysis using DNA microarray. Total RNA was extracted from ~10 mg of tissue samples using the miRNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The method of performing the DNA microarray analysis was previously described (13,14). Briefly, the ratio of the expression intensity between the tumor tissue (T) and the surrounding normal tissue (N) was calculated from the normalized values. The expression values for all probes were log (base 2) transformed before performing the statistical analysis.

Whole-exome sequencing (WES) analysis of the melanoma tissues using next-generation sequencing. WES analysis including mapping, variant calling and identification of somatic mutation were performed using the Ion Proton system with the Ion AmpliSeq™ Exome kit, Torrent Suite Software and Ion Reporter™ Server system (Thermo Fisher Scientific, Waltham, MA USA) as previously reported (12). Briefly, all the variants called by the variant caller were available. However, the data presented in SCC represent those variants considered to be of good quality, based on the filtering in which the sequences were discarded with a quality <30, variant allele frequency <10% or depth of coverage <20. Those mutations that were identified in tumor samples and not observed in matched normal samples were extracted as somatic mutations. Single-nucleotide variants (SNVs) of the total exonic mutations for each sequenced tumor included non-synonymous, synonymous, and indels/frameshift mutations. In the present study we focused on somatic SNVs. Additionally, Vogelstein driver gene mutation (15) profiling was investigated.

Immunohistochemistry. For the immune checkpoint protein staining, the anti-PD-L1 antibody (rabbit monoclonal, cat. 13684; 1:200 dilution) was purchased (Cell Signaling, Danvers, MA, USA). For the tumor-infiltrating lymphocyte (TIL) staining, anti-CD4 (mouse monoclonal, cat. MS-1528-S; 1:20 dilution) and anti-CD8 (mouse monoclonal, cat. MS-457-S; 1:50 dilution) antibodies (Thermo Fisher Scientific) were purchased and were used for the immunohistochemistry analysis. In each section stained with the various antibodies, 10 high-magnification (x200) fields were analyzed using WinROOF image-analyzing software (Mitani Corporation, Tokyo, Japan). The PD-L1 staining was evaluated as the percentage of tumor cells exhibiting positive membranous staining as follows: score 0, <1%; score 1, 1-5%; score 2, >5-50%; and score 3, >50% (16). The TIL level was assessed by a semi-quantitative estimation of the density of the $CD8^+$ T cells inside the tumor site as follows: score 0, no or sporadic $CD8^+$ T cells; score 1, moderate number of $CD8^+$ T cells; score 2, abundant number of $CD8^+$ T cells; and score 3, highly abundant number of $CD8^+$ T cells (17). The score that was most frequent in entire sections was assigned.

Statistical analysis. The differentially expressed genes derived from the 164 immune response-associated gene panels between the immunological parameter-positive and parameter-negative groups were identified using the volcano plot method. Each microarray probe was considered significantly differentially expressed between two groups of samples if they satisfied the following criteria: i) corrected t-test P-value <0.05; ii) a Benjamini-Hochberg false discovery rate (FDR) <0.1; and iii) a fold change >2.0 or below 1/2. Correlations between the immune response-associated gene expression and the clinicopathological features, including the survival data, were analyzed using an unpaired two-tailed t-test or a Spearman coefficiency test. Values of $P<0.05$ were considered significant. The relapse-free survival (RFS) was calculated from the date of the diagnosis until the date of distant relapse. The overall survival (OS) was calculated from the date of the diagnosis to the date of death from cancer. Follow-up was assessed from the date of the diagnosis to the last contact date with the event-free patients.

Results

PD-L1 and CD8 expression, Vogelstein driver genes mutations, and SNV number in melanoma tumors. PD-L1 expression was evaluated according to the criteria of the staining score, such that the scores of 1 and 2 were positive and a score of 0 was negative. Five cases were positive and 8 were negative for PD-L1 expression. According to the Vogelstein driver mutation number, the WES analysis revealed that 5 cases had ≥2 mutations, and 8 had <2 mutations. For the SNV number, 4 cases had ≥100 SNVs and 9 had <100. The CD8 expression level was high in 5 (scores 3 and 4) and low in 8 cases (scores 0-2) based on the IHC scoring denotations (Table I).

Table I. Melanoma patient list registered in Project HOPE.

Case	Age	Sex	Status	Relapse-free survival (M)	Overall survival (M)	PD-L1[b]	SNV no. (exon)	Vogelstein mutation no.	CD8[b]
MEL-001[a]	69	F	Alive	19	28	1	2712	12	2
MEL-002	79	F	Dead	-	-	2	84	0	3
MEL-003	41	F	Alive	24	24	0	15	0	2
MEL-004	50	M	Alive	22	22	1	35	1	4
MEL-005	60	M	Alive	22	22	0	31	0	1
MEL-006	31	F	Alive	19	19	0	45	3	2
MEL-007	88	F	Dead	4	8	1	737	8	3
MEL-008	78	M	Alive	19	19	0	88	1	4
MEL-009	81	M	Alive	17	17	0	30	0	1
MEL-010	85	M	Dead	5	16	1	297	3	1
MEL-011	58	F	Alive	12	14	0	114	1	0
MEL-012	82	F	Dead	3	4	0	69	2	3
MEL-013	58	M	Alive	10	10	0	39	0	2

[a]Metastatic lesion of the rib was used for analysis. [b]Immunohistochemical stain. HOPE, High-tech Omics-based Patient Evaluation; SNV, single nucleotide variant; F, female; M, male.

Table II. Upregulated gene list in PD-L1-positive melanomas.

Probe name	Gene symbol	FC	Log FC	Regulation	P-value
A_23_P412321	CCR5	3.948	1.981	Up	1.99×10^{-2}
A_23_P69310	CCRL2	5.753	2.524	Up	1.16×10^{-2}
A_23_P338479	CD274	11.825	3.564	Up	1.60×10^{-3}
A_23_P15394	CD68	3.930	1.975	Up	1.68×10^{-2}
A_24_P411561	HAVCR2	2.953	1.562	Up	2.43×10^{-2}
A_32_P351968	HLA-DMB	3.057	1.612	Up	4.15×10^{-2}
A_23_P112026	IDO1	9.284	3.215	Up	1.80×10^{-4}
A_23_P128919	LGALS3	4.402	2.138	Up	2.58×10^{-2}
A_24_P372223	MSR1	3.088	1.627	Up	2.53×10^{-2}
A_24_P274270	STAT1	4.126	2.045	Up	3.31×10^{-3}
A_23_P49338	TNFRSF12A	12.512	3.645	Up	2.56×10^{-2}
A_23_P51936	TNFRSF9	3.808	1.929	Up	3.06×10^{-2}
A_33_P3397763	TNFSF9	-5.673	-2.504	Down	2.84×10^{-2}

PD-L1, programmed death-ligand 1; FC, fold change.

Association of the immune response-associated gene expression with immunological parameters using a volcano plot. We previously established an immune response-associated gene panel, consisting of 164 genes (11). The association of the immune response-associated gene expression obtained by the GEP data from Project HOPE with PD-L1 expression, SNV number, Vogelstein driver gene mutation number and CD8 expression was investigated using a volcano plot analysis.

With regard to the PD-L1 expression, 12 immune response-associated genes were identified as upregulated genes in the PD-L1-positive melanomas, in which 6 genes were involved in T-cell suppression and 6 were related to T-cell activation (Fig. 1A and Table II). In addition, the VEGF gene alone was identified as an upregulated gene in high SNV number with >100 melanomas (Fig. 1B). Regarding the Vogelstein driver mutation number, in contrast, 18 immune response-associated genes were downregulated in the Vogelstein mutation high-number group. Notably, 9 genes involved in T-cell activation, such as CD3 (D, G and Z), CD40LG, STAT4, CCL5, TNFRSF4, TNFSF8 and TNFSF14, were identified (Fig. 1C and Table III). Notably, 14 immune response-associated genes were identified as upregulated genes in the TIL marker CD8-high melanomas, which were mostly correlated to T-cell activation favoring a Th1 response leading to tumor killing by CTLs (Fig. 1D).

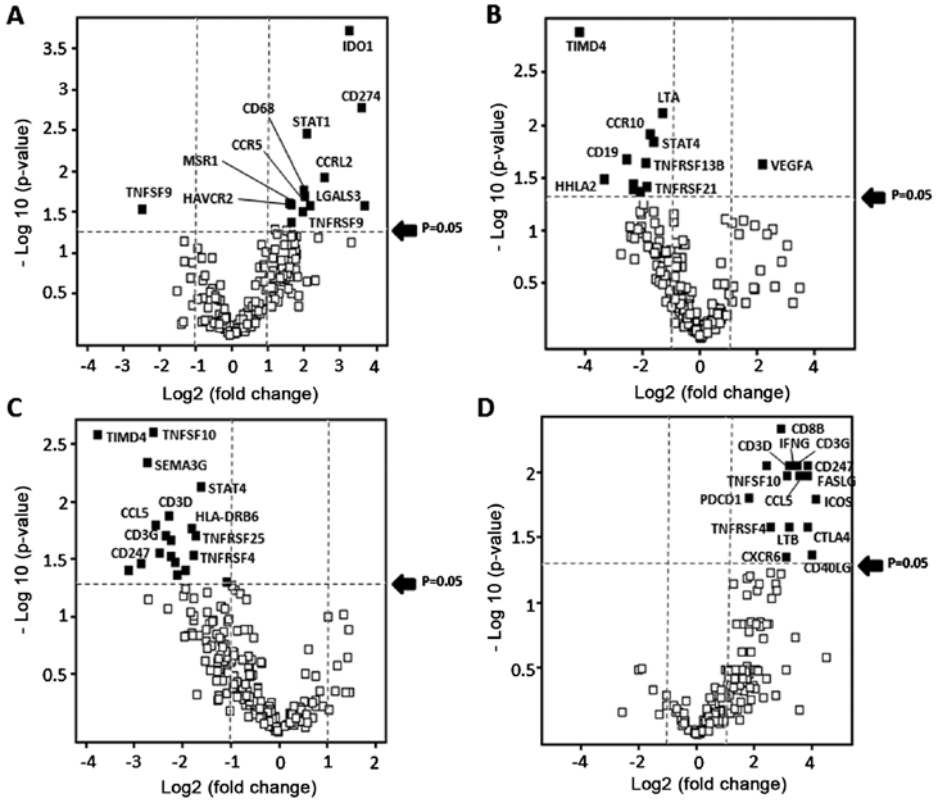

Figure 1. The association of the expression of the immune response-associated genes with PD-L1 expression, SNV number, Vogelstein driver gene mutation number and CD8 expression. The GEP data of a panel of 164 immune response-associated genes from 13 melanoma tumors were analyzed in terms of the association with various parameters using a volcano plot method. (A) PD-L1 expression. Five positive (IHC score ≥1) and 8 negative (IHC score 0) cases. (B) SNV number. Four positive (SNV no. ≥100) and 9 negative (SNV no. <100) cases. (C) Vogelstein mutation number. Five positive (mutation no. ≥2) and 8 negative (mutation no. <2) cases. (D) CD8 expression. Five positive (IHC score 3-4) and 8 negative (IHC score 0-2) cases. The horizontal dashed line represented a P-value of 0.05. The vertical dashed lines revealed 2- and 0.5-fold changes in gene expression. The closed squares represented differentially altered genes in expression, with more than a 2-fold difference. PD-L1, programmed death-ligand 1; SNV, single nucleotide variant; GEP, gene expression profiling; IHC, immunohistochemistry.

Table III. Downregulated gene list in driver mutation high melanomas.

Probe name	Gene symbol	FC	Regulation	P-value
A_24_P63380	BMPR1B	-4.37088	Down	4.24×10^{-2}
A_33_P3358923	BTLA	-4.76685	Down	2.12×10^{-2}
A_23_P152838	**CCL5**	-5.93301	Down	1.55×10^{-2}
A_23_P34676	**CD247 (CD3ζ)**	-5.64369	Down	2.71×10^{-2}
A_33_P3375541	**CD3D**	-4.90473	Down	1.29×10^{-2}
A_23_P98410	**CD3G**	-5.11053	Down	1.91×10^{-2}
A_33_P3250680	**CD40LG**	-7.29745	Down	3.38×10^{-2}
A_33_P3218980	ENTPD1	-3.87063	Down	3.87×10^{-2}
A_24_P169013	HLA-DRB6	-3.53737	Down	1.66×10^{-2}
A_33_P3248265	LTB	-4.80739	Down	2.93×10^{-2}
A_23_P6818	SEMA3G	-6.73986	Down	4.43×10^{-3}
A_23_P68031	**STAT4**	-3.11526	Down	7.24×10^{-3}
A_23_P7503	TIMD4	-13.6631	Down	2.52×10^{-3}
A_33_P3234530	TNFRSF25	-3.34808	Down	1.91×10^{-2}
A_33_P3286157	**TNFRSF4**	-3.47391	Down	2.84×10^{-2}
A_23_P121253	TNFSF10	-4.52915	Down	3.28×10^{-2}
A_21_P0000113	TNFSF10	-6.11132	Down	2.40×10^{-3}
A_24_P237036	**TNFSF14**	-8.744	Down	3.86×10^{-2}
A_23_P169257	**TNFSF8**	-2.1605	Down	4.88×10^{-2}

Bold indicates activated T-cell-associated genes. FC, fold change.

Table IV. Correlation of immune response-associated genes with survival time.

Overall survival		
Gene name	r-value	P-value
CCR6	0.6829	0.0295
CD27	0.7561	0.0114
CDH3	0.7744	0.0085
CXCR6	0.7561	0.0114
IL17RB	0.6646	0.0036
PDCD1	0.6525	0.0409
TNFRSF11A	0.9147	0.0002
ADAM12	-0.8721	0.0011
EDA2R	-0.8598	0.0014
GREB1	-0.7073	0.0221
IL6	-0.6829	0.0295
STAT5A	-0.7622	0.0014
TDO2	-0.6342	0.0489
TREM1	-0.7012	0.0239
Relapse-free survival		
Gene name	r-value	P-value
BTLA	0.7078	0.0221
B7H5	0.6585	0.0384
B7H7	0.7632	0.0102
CD27	0.8493	0.0019
CD3E	0.6893	0.0274
CD8B	0.7816	0.0076
CXCR6	0.8555	0.0016
FASLG	0.6401	0.0462
IL17RB	0.7571	0.0112
LAG3	0.7447	0.0135
PDCD1	0.7936	0.0061
TIMD4	0.7509	0.0123
TNFRSF11A	0.7755	0.0084
TNFRSF21	0.6647	0.0362
ADAM12	-0.7385	0.0147
EDA2R	-0.7016	0.0237
TREM1	-0.6524	0.0409

Bold indicates common genes in both overall and relapse-free survival.

Correlation of the immune response-associated gene expression with the survival time of the melanoma patients. The correlation of the expression of 164 immune response-associated genes with the overall and relapse-free survival time was investigated using a Spearman's rank-order correlation. Fourteen genes and 17 genes were significantly correlated with the overall and relapse-free survival time, respectively (Table IV). Eight genes, including CD27, CXCR6, IL17RB, PDCD1, TNFRSF11A, ADAM12, EDA2R and TREM1, were commonly identified in both the overall and relapse-free survival time groups, and 5 were positively correlated and 3 were negatively correlated.

Discussion

In the present study, we used a previously reported immune response-associated gene panel that consisted of 164 genes (11), and investigated the association of the expression of the gene panel with immunological and clinical parameters, such as: i) PD-L1 expression; ii) a high mutation load [single nucleotide variant (SNV) number]; iii) driver gene mutation; iv) CD8 expression; and v) survival time, using the genomic data from 13 melanoma patients registered in Project HOPE.

With advances in cancer genomic sequencing, specific gene signatures involved in the therapeutic response and prognosis have been reported, and their accuracy and efficiency have been investigated in various types of cancer, such as breast, stomach, non-small cell lung cancers and melanomas (18,19). However, few studies focusing on immune-related gene panels or signature identifications have been reported since the development of cancer genomic technologies such as next-generation sequencing. The identification of cancer-specific T-cell receptor (TCR) sequences has been attempted in immunological routine analyses (20), but not much success has been obtained. Small scale genetic studies focusing on renal cell cancer or polypoid precancerous colorectal lesions revealed that tumor-associated macrophage markers or TIL markers were involved in the prognosis or the progression of precancerous to cancerous lesions (21,22). However, Lee *et al* (23) obtained biopsy tissues from 55 triple-negative breast cancer patients treated with combined chemotherapy, and evaluated immune responses using the NanoString nCounter GX human immunology panel (579 immune-related genes), which demonstrated that a higher expression of cytotoxic molecules, TCR signaling pathway molecules, Th1 cytokines and B cell markers were associated with a pathological complete response (CR).

First, the association of the immune response-associated gene panel expression with the expression level of PD-L1 was investigated in the present study. Twelve immune response-associated genes were identified as upregulated in the PD-L1-positive melanomas; 6 of these genes were involved in T-cell suppression and 6 were related to T-cell activation. In particular, the 6 T-cell stimulation-related genes were: CCRL2 (attraction of TILs) (24); CD68 (M1 macrophage activation); CCR5 (T-cell migration); HLA-DMB (increase of $CD8^+$ TIL and IFN-γ level, and improvement of survival) (25); STAT1 (IFN-γ signal activation in T cells) (26) and TNFRSF9 (T-cell activation) (27). However, the others were T-cell inhibition-related genes, including; CD274 (PD-L1), IDO-1, HAVCR2 (TIM-3), LGALS3 (galectin-3), MSR1 and TNFRSF12A. Taube *et al* (28) reported similar results using a volcano plot of 11 melanoma patients, which demonstrated 12 upregulated genes in PD-L1-positive melanomas including 4 immuno-regulatory genes, such as CD274, PDCD1 (PD-1), LAG3 and IL-10. The upregulated gene profile in the PD-L1-positive melanomas in our study was similar to their analysis.

Second, the association of the immune response-associated gene panel expression with Vogelstein driver mutation number was investigated. Eighteen immune response-associated genes were downregulated in the Vogelstein mutation high-number (>2) group. Notably, 9 genes involved in T-cell activation, including CD3 (D, G and Z), CD40LG, STAT4, CCL5, TNFRSF4, TNFSF8

and TNFSF14, were identified. Among these, TNFRSF4 (OX40), TNFSF8 (CD30-L) and TNFSE14 (HVEM-L) are TNF ligand superfamily members and trigger T-cell stimulating signals by binding to their specific receptors. A constitutive signal activation, such as MAPK, STAT3, NF-κB, and β-catenin, in cancer cells induces an immunosuppressive effect that is mediated by the TGF-β, IL-6, IL-10 and VEGF produced by the cancer cells, resulting in regulatory T-cell and myeloid derived suppressor cell (MDSC) induction (29). Specifically, an STK11 mutation and RAS/MAPK activation were linked to CD3 gene down-regulation or a TIL reduction in the tumor (30,31) Additionally, Frederick *et al* (32) demonstrated that a BRAF inhibition was associated with an upregulation of melanoma antigen expression and a favorable tumor microenvironment through the reduction of immunosuppressive cytokines, such as IL-6 and IL-8. In the present study, an extensive immunosuppressive effect on the T-cell activation signal was ascertained, but the upregulation of melanoma antigens was not significant because of the small number of cases in the evaluation.

Third, the correlation of the expression of 164 immune response-associated genes with the overall and relapse-free survival time was investigated using a Spearman's rank-order correlation. Eventually, 8 genes, such as CD27, CXCR6, IL17RB, PDCD1, TNFRSF11A, ADAM12, EDA2R and TREM1, were commonly identified in both the overall and relapse-free survival time groups, of which 5 were positively correlated and 3 were negatively correlated. Briefly, the survival-correlated gene profiling was as follows: CD27 (expressed on $CD8^+$ TIL was associated with a good prognosis) (33); CXCR6 (the CXCR6/CXCL16 axis in the tumor was associated with a TIL increase and a good prognosis) (34); IL17RB (a higher HOXB13-to-IL17RB ratio was linked to a worse outcome) (35); TNFRSF11A (RANK upregulation may be linked to mammary tumorigenesis in BRCA1-mutant carriers) (36); ADAM12 [an aggressive ovarian cancer marker was associated with a TGF-β-induced epithelial to mesenchymal transition (EMT)] (37); EDA2R (highly expressed in ovarian cancer and was associated with a poor prognosis); and TREM1 (induced a proinflammatory and protumor microenvironment and was associated with a poor prognosis) (38). Based on these observations, the protein expression of the 8 markers, using previously resected melanoma tissues, warrants future investigation, and the specific association of the protein expression with the survival data based on a Kaplan-Meier analysis should be precisely performed.

Finally, in the present study, we investigated the association of the expression of the immune response-associated gene panel with various parameters, mainly PD-L1 and CD8 expression, driver gene mutation and survival time, and several gene signatures involved in patient prognosis were identified. These results revealed that cancer genomic data may be associated with specific immunological gene signatures closely linked to the immunological status in the tumor microenvironment, which could contribute to the development of specific cancer immunotherapies for tailored medicine called precision immunotherapy (39).

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