Identification of tumor microenvironment-associated immunological genes as potent prognostic markers in the cancer genome analysis project HOPE

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Abstract. Project High-tech Omics-based Patient Evaluation (HOPE), which used whole-exome sequencing and gene expression profiling, was launched in 2014. A total of ~2,000 patients were enrolled until March 2016, and the survival time was observed up to July 2019. In our previous study, a tumor microenvironment immune type classification based on the expression levels of the programmed death-ligand 1 (PD-L1) and CD8B genes was performed based on four types: A, adaptive immune resistance; B, intrinsic induction; C, immunological ignorance; and D, tolerance. Type A (PD-L1$^+$ and CD8B$^+$) exhibited upregulated features of T helper 1 antitumor responses. In the present study, survival time analysis at 5 years revealed that patients in type A had a better prognosis than those in other categories [5 year survival rate (%); A (80.5) vs. B (73.9), C (73.4) and D (72.6), P=0.0005]. Based on the expression data of 293 immune response-associated genes, 62 specific genes were upregulated in the type A group. Among these genes, 18 specific genes, such as activated effector T-cell markers (CD8/CD40LG/GZMB), effector memory T-cell markers (PD-1/CD27/ICOS), chemokine markers (CXCL9/CXCL10) and activated dendritic cell markers (CD80/CD274/SLAMF1), were significantly associated with a good prognosis using overall survival time analysis. Finally, multivariate Cox proportional hazard regression analyses of overall survival demonstrated that four genes (GZMB, HAVCR2, CXCL9 and CD40LG) were independent prognostic markers, and GZMB, CXCL9 and CD40LG may contribute to the survival benefit of patients in the immune type A group.

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Abbreviations: PD-1, programmed death-1; PD-L1, programmed death-ligand 1; WES, whole-exome sequencing; GEP, gene expression profiling; SNV, single nucleotide variant; CTL, cytotoxic T lymphocytes; TME, tumor microenvironment; TMB, tumor mutational burden; OST, overall survival time

Key words: TME, immune response-associated gene, prognostic marker, immune type classification, project High-tech Omics-based Patient Evaluation

Introduction

Since the development of immune checkpoint blockade cancer therapy, many clinical trials of immune checkpoint therapy combined with conventional targeted therapy against

solid cancers have been performed, and this treatment has achieved great success in the cancer treatment field as a novel immunotherapy (1-3). With advances in clinical cancer immunotherapeutic regimens, closely associated tumor-related parameters have been intensively investigated. These parameters are thought to be linked to the efficacy of immune checkpoint blockade therapy and the prognosis of cancer patients (4-7). However, in the tumor microenvironment, there are many factors, such as genetic, immunological (cellular or humoral), and metabolic factors, that have been demonstrated to be involved in the immunosuppressive mechanism. For example, as cellular factors, regulatory effector T cells, myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and cancer-associated fibroblasts (CAFs) have been reported to exhibit protumor immunosuppressive actions (8-10).

Moreover, immune-type classifications that can contribute to the prediction of immune checkpoint blockade efficacy and the prognosis of cancer patients have been performed by several researchers using three main types of immunological features: PD-L1 expression level, tumor-infiltrating lymphocyte (TIL) status and tumor mutational burden (TMB) (11-16). PD-L1 is a major immune checkpoint molecule that is expressed on tumor cells or associated macrophages and is supposed to inhibit activated T cell function via PD-1/PD-L1 binding (17,18). Meanwhile, some researchers have demonstrated that the simple combination of PD-L1$^+$ and TIL$^+$ (CD8$^+$) may predict a good response to immune checkpoint blockade (11,12). Others have reported that TMB is a genuine biomarker for the prediction of immune checkpoint blockade efficacy (14).

Previously, our group performed an immunological classification based on PD-L1 and CD8B gene expression levels and demonstrated that the PD-L1+ and CD8B+ groups were associated with the upregulation of cytotoxic T lymphocyte (CTL) killing-associated genes, T cell activation genes, antigen-presentation genes and dendritic cell (DC) maturation genes, and promoted T helper 1 (Th1) antitumor responses (19). However, there are few immune-type classification studies that directly evaluated cancer patient prognosis.

In the present study, we verified that the PD-L1$^+$CD8B$^+$ group (type A) was associated with a better prognosis [5-year overall survival time (OST)] than the other types. In addition, we identified prognostic factors responsible for the survival benefit of patients in type A based on 293 immune response-associated gene expression datasets.

Materials and methods

Patient characteristics and study design. The Shizuoka Cancer Center launched Project HOPE in 2014 using multiomics analyses including whole exome sequencing (WES) and gene expression profiling (GEP). Ethical approval for the HOPE study was obtained from the Institutional Review Board of Shizuoka Cancer Center (authorization no. 25-33). In total, 1,763 patients with tumors were enrolled until March 2016 and the survival time was observed up to July 2019.

Clinical specimens. Tumor tissue samples weighing more than 0.1 g and with a tumor content greater than 50% were dissected along with surrounding normal tissue samples by pathologists.

GEP and WES analysis. DNA and RNA isolation and the GEP and WES analyses were performed as described previously (20). RNA samples with an RNA integrity number ≥6.0 were used for microarray analysis. Labeled samples were hybridized to the SurePrint G3 Human Gene Expression 8x60 K v2 Microarray (Agilent Technologies). Microarray analysis was performed in accordance with the MIAME guidelines. For DNA data analysis, somatic mutations were identified by comparing data from tumor and corresponding blood samples. Mutations in 138 known driver genes were defined as those identified as pathogenic in the ClinVar database. Vogelstein *et al* (21) demonstrated that 138 genes, when altered by intragenic mutations, can promote or drive tumorigenesis. A most of tumors including colorectal cancers contain two to eight of these 'driver gene' mutations and the remaining mutations are passengers that do not contribute to tumorigenesis directly. Thus, these 138 driver mutations are accepted as relevant genes to the tumorigenesis (21). Single nucleotide variants (SNVs) of the total exonic mutations for each sequenced tumor included nonsynonymous, synonymous, and indel/frameshift mutations.

Renewal of the immune response-associated gene panel. The immune response-associated gene panel was described previously (22). In the present study, the gene panel was renewed by adding 119 immunological genes (293-gene panel) as shown in Table I. The panel consisted of 114 antigen-presenting cell (APC), T cell and natural killer cell receptor (NKR) genes; 48 cytokine signal and metabolic genes; 48 tumor necrosis factor (TNF) and TNF receptor superfamily genes; 23 regulatory T cell-associated genes; and 60 IFN-g pathway genes.

Statistical analysis. Based on the expression levels of the PD-L1 and CD8B genes, we classified all 1,763 tumors enrolled in the HOPE project into 4 immune types: type A, PD-L1$^+$CD8B$^+$; type B, PD-L1$^+$CD8$^-$; type C, PD-L1$^-$CD8B$^-$; and type D, PD-L1$^-$CD8B$^+$ as described previously. A comparative analysis of the survival times between group A and the other groups was performed using the Kaplan-Meier method and Cox proportional hazards regression model. The upregulated genes derived from the 293-immune response-associated gene panel between tumor microenvironment (TME) immune type A and other types were identified using the volcano plot method with Benjamini-Hochberg correction. Upregulated immune response-associated genes with >2-fold expression differences ($P<0.05$) were identified. The heatmap expression data of upregulated genes in the immune type A group were investigated using GeneSpring GX software version 13.1.1 (Agilent Technologies). The association of upregulated gene expression levels with the OST was examined using the Kaplan-Meier method. A comparative analysis of the survival times between patients with low expression (less than the median) and patients with high expression (more than the median) of the identified genes in group type A (referred as to group A) was performed by the log-rank test using EZR software and Microsoft Excel. Regarding probable prognosis-associated genes identified in group A, the significance of these genes was analyzed using a multivariate Cox proportional hazards regression model with EZR software (23). Values of $P<0.05$ denoted statistically significant differences.

Table I. Immune response-associated genes list.

Groups	Genes	No. of genes
APC, T cell and NKR genes	CD80, CD86, CD274 (PD-L1), PDCD1LG2 (PD-L2), ICOSLG, CD276, VTCN-1, C10orf54, B7H6, HHLA2, LGALS9, SIRPB1, TREM1, CLEC5A, SIGLEC14, CD68, CD204(MSR1), HLA-DPA, HLA-DQA, HLA-DRA, HLA-DRB1, HLA-DQA2, CD19, CD20, CD38, CD138, CD28, CTLA4, CD279 (PD-1), ICOS, BTLA, SLAMF1, HAVCR1, HAVCR2, TIMD4, TREML2, LAG3, CD247 (CD3zeta), CD4, CD8A, CD8B, CD25, FOXP3, CCR4, CD56 (NCAM1), CD3D, CD3G, CD3E, HLA-A, HLA-B, HLA-C, HLA-E, MICA, MICB, ULBP1, ULBP2, ULBP3, RAET1E, NKp44L, CLEC2D, CLEC12B, CDH1, CDH2, CDH3, CDH4, CD83, CD11b, CD11c, CD209, TIGIT, CD155, CD200, CD200R, GZMB, PRF1, CD44, CD45, CD62L, CCR7, CXCR3, CXCR4, CD69, BCL2, CD122, CD127, CD16, CD314 (NKG2D), CD335 (NCR1), TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10, DDX58, IFIH1, DHX58, NOD1, NOD2, CLEC4E, CLEC6A, CLEC7A, STING, TOX, TCF7, B2MG, TAP1, TAP2, NT5E (CD73), ADRA2A	114
Cytokine signal and metabolic genes	TGFB1, TGFB2, TGFB3, TGFBR1, TGFBR2, VEGFA, IFNA1, IFNA2, IFNB1, IL2, IL4, IL6, IFNG, IL10, IL12A, IL17A, IL23A, IDO1, ARG1, NOS2, PTGS2, AHR, TDO2, JAK2, STAT1, STAT3, STAT4, STAT5, STAT6, SOCS1, VCAM1, CCL2, CCL3, CCL4, CCL5, CCL19, CCL21, CCL22, CXCL5, CXCL8, CXCL9, CXCL10, CXCL12, CXCR2, CSF1, CSF2, CSF3, CSF1R	48
TNFSF and TNFRSF	TNFSF1 (LTA), TNFSF2 (TNF), TNFSF3 (LTB), TNFSF4, TNFSF5 (CD40LG), TNFSF6 (FASLG), EDA, TNFSF7 (CD70), TNFSF8, TNFSF9, TNFSF10, TNFSF11, TNFSF12, TNFSF13, TNFSF13B, TNFSF14, TNFSF15, TNFSF18, TNFRSF1A, TNFRSF1B, EDAR, TNFRSF3 (LTBR), TNFRSF4, TNFRSF5 (CD40), TNFRSF6 (FAS), TNFRSF6B, TNFRSF7(CD27), TNFRSF8(CD30), TNFRSF9, TNFRSF10A, TNFRSF10B, TNFRSF10C, TNFRSF10D, TNFRSF11A, TNFRSF11B, TNFRSF12A, TNFRSF13B, TNFRSF13C, TNFRSF14, TNFRSF16(NGFR), TNFRSF17, TNFRSF18, TNFRSF19, TNFRSF19L(RELT), TNFRSF21, TNFRSF25, TNFRSF27 (EDA2R)	48
Regulatory T cell-associated genes	SEMA3G, LGALS3, ENTPD1 (CD39), CCR6, CCL20, IL12RB2, CCR10, ANXA2, IL17RB, ADAM12, TMEM45A, LRRC32, LOXL1, GREB1, HRH4, CCR5, BMPR1B, SFRP1, LAMA2, ITGB1, CPE, MKI67, CDCA3	23
IFN-γ pathway genes	IFIT1B, IFNA21, IFNW1, IFNA14, IFNA4, IFNA5, IFNA6, IFNA8, IFNE, IFIT1, IFNK, CNTFR, IFIT2, IFIT3, IL10RA, IL11RA, IL20RA, CREB3, IL12B, IL31RA, IL7R, IFI30, IFNGR1, IFRD1, IFRD2, IL22RA2, IL5RA, IRF1, IRF8, IRGM, JAK1, MX1, OAS1, PIK3CA, PRKCD, PYH1N1, PIAS4, EBI3, IFI27, IFNAR1, IFNAR2, IFNGR2, IL10RB, IL21R, IL28A, IL28RA, IL29, IL4R, IL6R, IRF2BP1, IRF3, IRF4, IRF5, LEPR, MPL, SP110, STAT2, TBX21, TYK2, SOCS3	60
Total		293

APC, antigen-presenting cell; NKR, natural killer cell receptor; TNFSF, TNF super-family; TNFRSF, TNF receptor super-family.

Table II. Association of immunological and genetic features with overall survival.

Group	Cohort (case no./5yrOS)	P-value
Genetic mutations		
Vogelstein	MT (1084/77.3%) vs. WT (679/74.6%)	0.184
TP53	MT (729/74.5%) vs. WT (1034/77.4%)	0.206
KRAS	MT (299/77.7%) vs. WT (1464/75.9%)	0.431
EGFR	MT (107/73.7%) vs. WT (1656/76.3%)	0.215
PIK3CA	MT (169/80.5%) vs. WT (1594/75.9%)	0.625
BRAF	MT (64/77.1%) vs. WT (1699/76.2%)	0.912
TMB number	>20 (83/81.7%) vs. <20 (1679/76.0%)	0.512
Gene amplification[a]		
All 64 genes[b]	Yes (575/75.9%) vs. No (833/75.9%)	0.858
EGFR	Yes (61/75.4%) vs. No (1347/75.9%)	0.746
HER2	Yes (33/71.3%) vs. No (1375/76.0%)	0.530

[a]Gene amplification, fold-change in expression ≥5 and copy number ≥6. [b]64 amplified gene list was reported previously (20). Comparison of MST between cohorts was performed using the log-rank test. P<0.05 denote statistically significant differences. 5yrOS, 5-year overall survival rate; MT, mutated; WT, wild-type; TMB, tumor mutation burden number.

Results

Association of the overall survival time with immune types. The 1,763 pairs of tumors and adjacent normal tissues derived from different cancer types were classified into 4 immune types based on the expression levels of the PD-L1 and CD8B genes. The patient numbers with different cancer types were described previously (17). The proportions of TME immune types A, B, C and D were 39.3, 26.5, 19.1 and 15.1%, respectively. Survival time analysis at 5 years revealed that group A had a better prognosis than the other groups [5 year survival rate (%); A (80.5) vs. B (73.9), C (73.4) and D (72.6), P=0.0005] (Fig. 1).

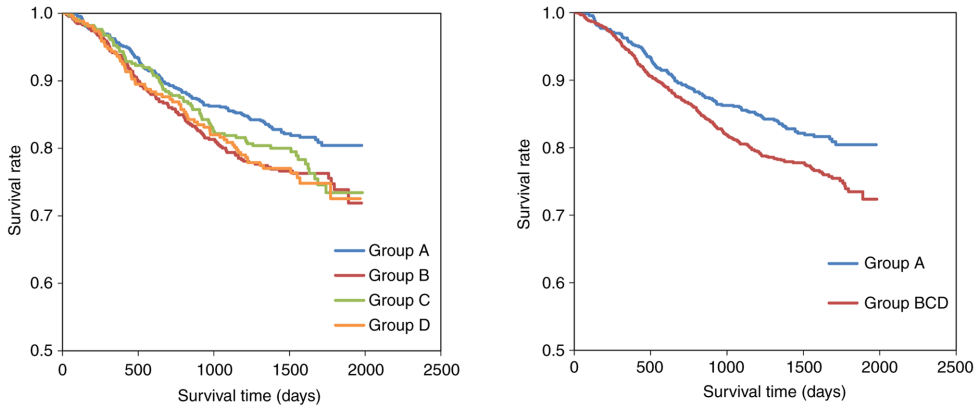
Association of genetic mutations and immunological surface markers with overall survival. The characteristics of genetic mutations, including Vogelstein driver mutations and SNVs, and gene amplification were described previously (19). The association of the genetic mutation status of driver gene mutations, such as TP53, KRAS, EGFR, PIK3CA and BRAF mutations, or gene amplification with the OST was investigated using the log-rank test. There was no significant association of genetic parameters with the OST (Table II).

The identification of upregulated immune response-associated genes in immune type A compared with the other types. Based on the expression profile of the 293-immune response-associated gene panel, 62 upregulated immune response-associated genes (more than 2-fold and P-value 1.0E-50) were identified using volcano plots (Fig. 2).

Comparison of upregulated genes among immune types or between the poor prognosis and good prognosis cohorts. The heatmap expression data of 62 upregulated genes in group A were compared with those of the other groups. Interestingly, in group A, T cell effector activation genes (CXCL9, CXCL10, and TNFRSF9) and CTL killing genes (GZMB, CD16) showed high expression, while immune checkpoint genes such as CTLA4 and TIGIT also showed high expression levels. In contrast, in group C, T cell effector activation genes (ICOS, CD69, and CD40LG) and Th1 cytokine genes (IFNG and TNF) exhibited low expression (Fig. 3). Additionally, the upregulated T cell activation genes identified in group A showed a tendency of higher expression levels in the better survival cohort than in the poorer survival cohort, as shown in Fig. 4.

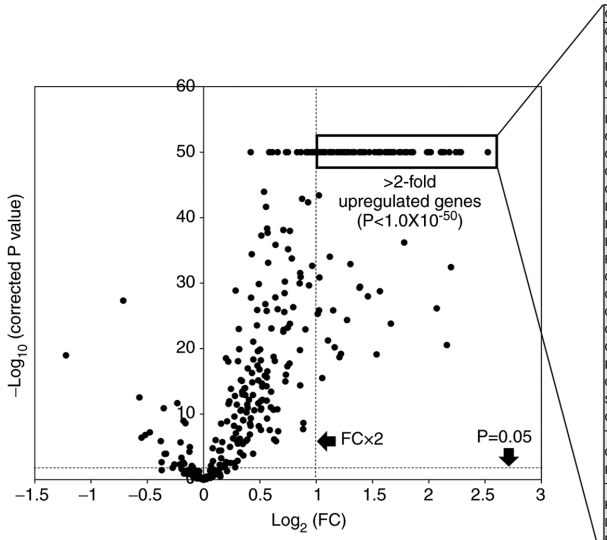
Association of the upregulated gene expression level with the overall survival time. The association of 62 upregulated genes in group A with the OST was analyzed by the log-rank test using EZR software. Ultimately, 18 genes were found to be significantly associated with prognosis (Table III). Memory T cell markers such as PD-1, CD27 and ICOS, as well as activated effector T cell genes (GZMB, CXCL10 and CD40LG) and mature DC marker genes (CD80 and SLAMF1), were selected as prognostic factors. Interestingly, immune checkpoint marker genes, such as HAVCR2 and TIGIT, were also verified as prognostic markers; however, the HAVCR2 gene was demonstrated to be a poor prognostic marker, although it was upregulated in group A.

Identification of probable prognostic genes using multivariate Cox hazards regression analysis. To evaluate the prognostic value of the genes, 18 probable prognostic genes identified using the Kaplan-Meier method from 62 upregulated genes in group A were analyzed by the Cox proportional hazards regression model. In particular, the multivariate analysis demonstrated that four upregulated genes, namely, GZMB, HAVCR2, CXCL9 and CD40LG, maintained their significance (P<0.05), as shown in Table IV. The survival curves of these four significant genes were drawn with the Kaplan-Meier method, and the OST was compared between the group that was higher-than-the median-level and the group



	N	5 yrOS (%)	HR (95% CI)	*P* value
A	692	80.5%	Ref	Ref
B	467	73.9%	1.386 (1.075–1.788)	0.0119*
C	337	73.4%	1.278 (0.960–1.699)	0.0922
D	267	72.6%	1.414 (1.048–1.909)	0.0234*
BCD	1071	73.5%	1.358 (1.097–1.683)	0.005**

Figure 1. Evaluation of the OS time in 1,763 patients with cancer registered in the HOPE project. Survival time analysis at 5 years revealed that group A had a better prognosis than the other groups. A comparative analysis of the survival times between group A and the other groups was performed using the Kaplan-Meier method and Cox proportional hazards regression model. The OS analysis indicated a significant survival benefit at 5 years for group A. *P<0.05 and **P<0.01. Number of cases in group A (n=692), group B (n=467), group C (n=337) and group D (n=267). The number of cases with different types of cancer was as follows: 107 breast, 601 colorectal, 27 skin, 25 esophageal, 248 stomach, 49 uterine and ovarian, 69 bile duct and pancreatic, 152 head and neck, 98 liver, 4 brain, 14 bone, 348 lung and 21 kidney cancer. OS, overall survival; HR, hazard ratio; HOPE, High-tech Omics-based Patient Evaluation.



Gene Symbol	ProbeName	FC
CXCL9	A_23_P18452	5.751
CXCL10	A_24_P303091	4.874
IFNG	A_23_P151294	4.539
GZMB	A_23_P117602	4.401
TIGIT	A_33_P3342056	4.335
ICOS	A_23_P371215	4.310
CCR7	A_23_P343398	4.047
CTLA4	A_33_P3396139	4.014
CD8B	A_23_P357881	3.968
CD274	A_23_P338479	3.960
IL7R	A_23_P404494	3.582
IL2RB	A_24_P203000	3.579
IL21R	A_24_P227927	3.446
FASLG	A_23_P369815	3.365
CD38	A_23_P167328	3.348
CD69	A_23_P87879	3.331
CXCR3	A_23_P114299	3.229
CCL5	A_23_P152838	3.195
CD3G	A_23_P98410	3.159
IRF4	A_23_P214360	3.149
CD3D	A_33_P3375541	3.117
SELL	A_33_P3400273	3.096
CD247	A_23_P34676	3.072
TNFRSF9	A_23_P51936	3.056
CCL4	A_23_P207564	2.975
IL2RA	A_23_P127288	2.963
TNFSF14	A_24_P237036	2.950
KLRK1	A_24_P193093	2.921
LTB	A_33_P3248265	2.864
FCGR3A	A_23_P200728	2.771
BTLA	A_33_P3358923	2.771

Gene Symbol	ProbeName	FC
PRF1	A_23_P1473	2.698
PTPRC	A_33_P3364811	2.660
CXCR4	A_23_P102000	2.657
CD27	A_23_P48088	2.628
TBX21	A_23_P141555	2.619
CD40LG	A_33_P3250680	2.608
CCL3	A_33_P3316273	2.534
IRF1	A_23_P41765	2.526
CCR5	A_23_P412321	2.497
IRF8	A_33_P3343120	2.445
TLR8	A_23_P73837	2.441
TNF	A_23_P376488	2.428
PDCD1	A_23_P136405	2.388
IFI30	A_23_P153745	2.367
HLA-DQA1	A_33_P3293049	2.328
ITGAX	A_23_P312132	2.294
HLA-DQA2	A_24_P852756	2.276
STAT1	A_24_P274270	2.225
LAG3	A_23_P116942	2.192
STAT4	A_23_P68031	2.188
CLEC7A	A_24_P235988	2.171
CD80	A_24_P320033	2.167
TAP1	A_23_P59005	2.116
HLA-DRA	A_32_P87697	2.106
CD83	A_23_P70670	2.092
CD3E	A_23_P416747	2.052
TNFRSF4	A_33_P3286157	2.047
SOCS1	A_23_P420196	2.024
SLAMF1	A_23_P62647	2.012
HAVCR2	A_24_P411561	2.005
NOD2	A_23_P420863	2.004

Figure 2. Identification of upregulated immune response-associated genes in tumor microenvironment immune type A compared with other types. A total of 62 upregulated immune response-associated genes with >2-fold expression differences were identified using volcano plots with Benjamini-Hochberg correction. The upregulation of all listed genes denoted statistically significant differences (P<0.05). FC, fold-change.

that was lower-than-the median-level, as shown in Fig. 5. The upregulation of GZMB, CXCL9 and CD40LG gene expression might be linked to better prognosis in group A patients.

Discussion

With advances in genome analysis technologies such as NGS- and single-cell RNA sequencing, probable immunological factors belonging to the TME and associated with prognosis have been more intensively, specifically and accurately investigated (24-26). Beyond the already-known TME factors that might be responsible for the efficacy of cancer immunotherapy, such as positive PD-L1 expression, a high mutational burden and an advanced TIL status, more specific and dynamic biomarkers associated with the immune response have been reported (27-29). Recently, Kumagai *et al* demonstrated using cytometry by time of flight (CyTOF) analysis based on single-cell RNA-seq that a balance between

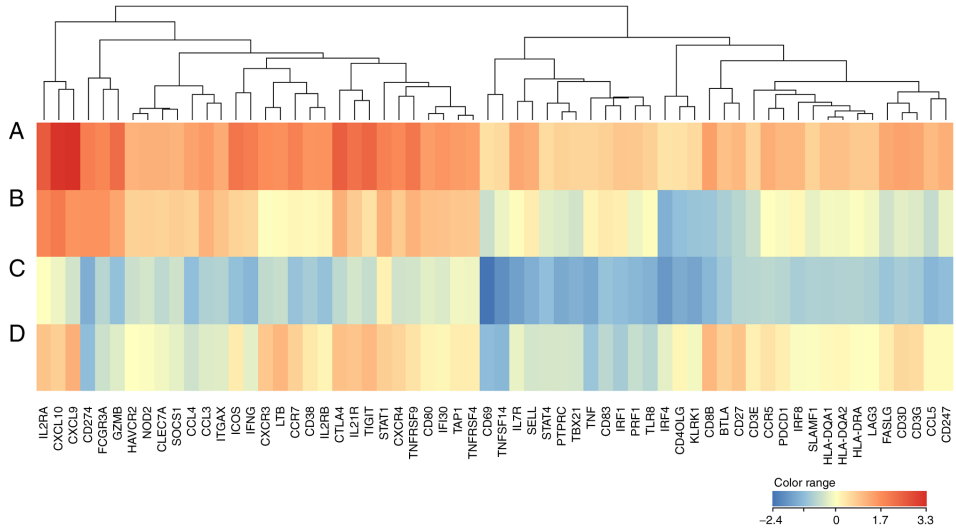


Figure 3. Hierarchical clustering analysis of 62 upregulated genes in each immune group. Each row in the matrix represents the expression level of a gene feature in an individual group. The red and blue colors in the panel reflect the relative expression level of the gene, as indicated in the color scale (log_2-transformed scale). Group A (n=692), group B (n=467), group C (n=337) and group D (n=267).

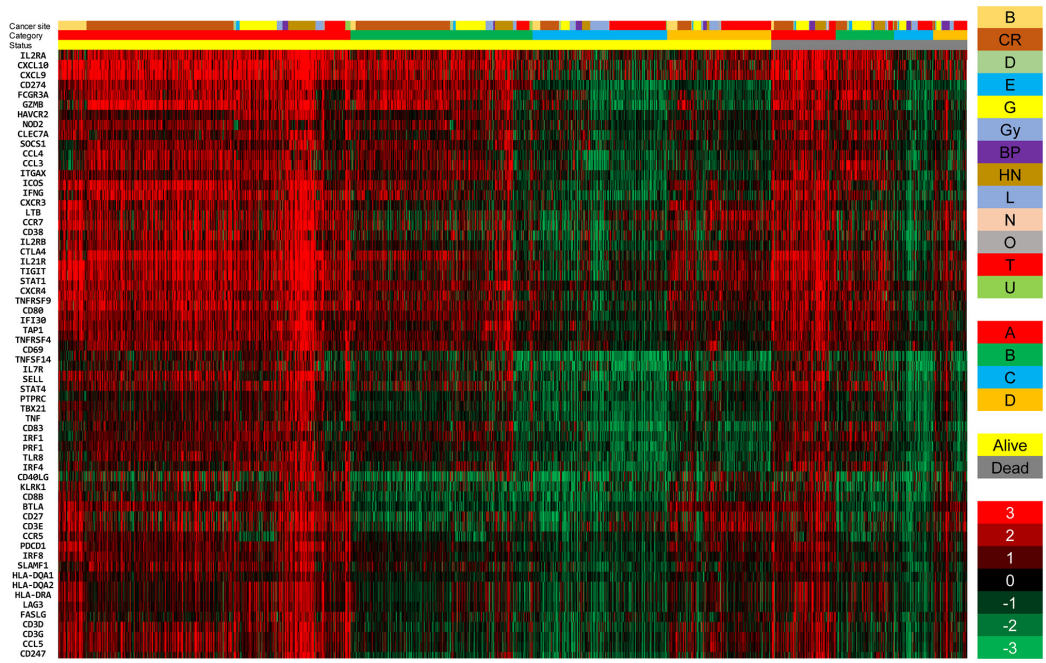


Figure 4. Comparison of the expression levels of 62 upregulated genes between alive and deceased patients with cancer. The two cohorts of patients were divided into 4 immune groups, and classified into 13 histological types. The data are presented in matrix format, where each row represents an individual case, and each column represents a gene. Each cell in the matrix represents the expression level of a gene in an individual case. The red and green colors reflect the gene expression levels, as indicated in the color scale (log_2-transformed scale) in the bottom right corner. B, breast; CR, colorectal; D, skin; E, esophageal; G, stomach; Gy, uterine and ovarian; BP, bile duct and pancreatic; HN, head and neck; L, liver; N, brain; O, bone; T, lung; and U, kidney.

PD-1$^+$CD8$^+$ T cells and PD-1$^+$CD4$^+$FoxP3$^+$ Treg cells is a critical determinant of the response to anti-PD-1/PD-L1 blockade therapy (29).

Previously, we reported an efficient immunological classification based on PD-L1 and CD8B gene expression levels and demonstrated that immune type A (PD-L1$^+$CD8B$^+$) was associated with the Th1 T cell and NK cell activation pathways, dendritic cell maturation and cancer-apoptosis activation signals and showed the highest score in immune-activation signaling pathways by means of Ingenuity Pathways Analysis (IPA) software (19). Similar studies have been conducted that showed antitumor immunological features in PD-L1+CD8+ cohort (11,12).

However, there have been few studies that have performed a long-term follow-up of overall survival in cancer patients belonging to the immune type classifications described above. Ock *et al* classified similarly solid tumors into specific immune types based on PD-L1 and CD8 gene expression data derived from The Cancer Genome Atlas (TCGA) database and compared the survival time between

Table III. Probable prognostic genes identified from 62 upregulated genes.

Probe name	Fold-change	Gene symbol	5yrOS (%)[a] Positive. vs. Negative	Log-rank P-value
A_23_P117602	4.401	GZMB	80.7 vs. 71.7	$1.44x10^{-4}$
A_24_P411561	2.005	HAVCR2	74.1 vs. 78.3	$2.03x10^{-3}$
A_23_P371215	4.31	ICOS	80.5 vs. 71.9	$2.14x10^{-3}$
A_23_P18452	5.751	CXCL9	80.5 vs. 71.8	$3.06x10^{-3}$
A_23_P420196	2.024	SOCS1	79.6 vs. 72.8	$3.44x10^{-3}$
A_23_P136405	2.388	PDCD1	80.3 vs. 72.1	$3.6x10^{-3}$
A_24_P303091	4.874	CXCL10	80.5 vs. 71.9	$4.76x10^{-3}$
A_23_P98410	3.159	CD3G	79.8 vs. 72.7	$1.47x10^{-2}$
A_23_P420863	2.004	NOD2	79.1 vs. 73.3	$1.82x10^{-2}$
A_33_P3250680	2.608	CD40LG	78.6 vs. 74.0	$2.52x10^{-2}$
A_33_P3375541	3.117	CD3D	79.7 vs. 72.7	$2.6x10^{-2}$
A_23_P62647	2.012	SLAMF1	79.7 vs. 72.6	$2.6x10^{-2}$
A_24_P320033	2.167	CD80	79.2 vs. 73.2	$2.96x10^{-2}$
A_23_P48088	2.628	CD27	79.9 vs. 72.7	$3.31x10^{-2}$
A_23_P416747	2.052	CD3E	78.9 vs. 73.6	$3.64x10^{-2}$
A_33_P3342056	4.335	TIGIT	79.3 vs. 73.2	$4.16x10^{-2}$
A_23_P338479	3.96	CD274	78.6 vs. 73.8	$4.33x10^{-2}$
A_23_P41765	2.526	IRF1	79.2 vs. 73.0	$4.53x10^{-2}$

[a]The 5yrOS between positive (higher expression than the median level) and negative (lower expression than the median level) groups were compared using the log-rank test using EZR software. Ultimately, 18 genes were found to be significantly associated with prognosis of patients with cancer. Only the HAVCR2 gene demonstrated a negative association with prognosis. 5yrOS, 5-year overall survival.

Table IV. Cox proportional hazards regression analysis of overall survival in upregulated genes.

Variable	Hazard ratio (95% CI)	P-value
GZMB	0.628 (0.496-0.795)	$1.11x10^{-4}$
HAVCR2	1.848 (1.479-2.309)	$6.63x10^{-8}$
CXCL9	0.778 (0.613-0.988)	0.0393
CD40LG	0.792 (0.642-0.977)	0.0292

From probable prognosis-associated genes identified in group A, the significance of those genes was analyzed using multivariate Cox proportional hazards regression model in the EZR software. $P<0.05$ denoted statistically significant differences.

immune types; however, the temporary difference in 3-year survival time in type A finally disappeared in the 5-year comparison (12).

In the current study, we followed 1,763 patients with tumors up to 70 months after registration in the project HOPE study. Survival time analysis at 5 years revealed that group A had a better prognosis than the other groups, as shown in Fig. 1. There are some concerns regarding the temporary results of the present survival analysis: i) Miscellaneous cancer patients across various histology groups were included, and ii) there were various clinical courses, including different types of therapies and response statuses. However, despite different clinical courses in individual patients, the immunological status at cancer diagnosis can be determined temporarily in terms of the OST, and could be a reference parameter for therapeutic design because some immunological mechanisms are involved in tumor regression after or even during chemo- and radiation therapy (30-33).

In the present study, the impressive findings were that memory T cell markers (central ~ effector memory), such as PD-1, CD27 and ICOS, were selected as prognostic factors. In addition to effector-activated CTLs and NK cells, memory marker$^+$ T cells should be considered crucial factors because i) PD-1$^+$ T cells can achieve a good balance between good and poor responses by immune checkpoint blockade (27), and ii) effector memory T cells that proliferate by the stimulation of antigen-presenting cells, can be differentiated into activated effector CTLs (34). Another important observation was that T cell exhaustion marker genes such as HAVCR22 (TIM3) and TIGIT were included as prognostic markers. However, HAVCR2 was found to be a negative prognostic marker, suggesting that it did not contribute to the good prognosis of patients in immune group A. Very recently, Simon *et al* demonstrated that a high frequency of the PD-1$^+$TIGIT$^+$ (double-positive) CD8$^+$ T cell subset in peripheral blood can be a good predictive marker for a good response to anti-PD-1 therapy (35). Therefore, these cells should be prolonged by anti-PD-1/PD-L1 blockade to maintain the antitumor effect, which could contribute to the good prognosis in cancer patients belonging to immune type A.

Additionally, based on prognostic factor profiling in immune group A, the upregulation of the CD80, CD274 and SLAMF1 (36) genes might suggest the presence of mature dendritic cells in the TME. Interestingly, Schetters *et al* demonstrated that anti-PD-1 immune checkpoint blockade induced mature monocyte-derived

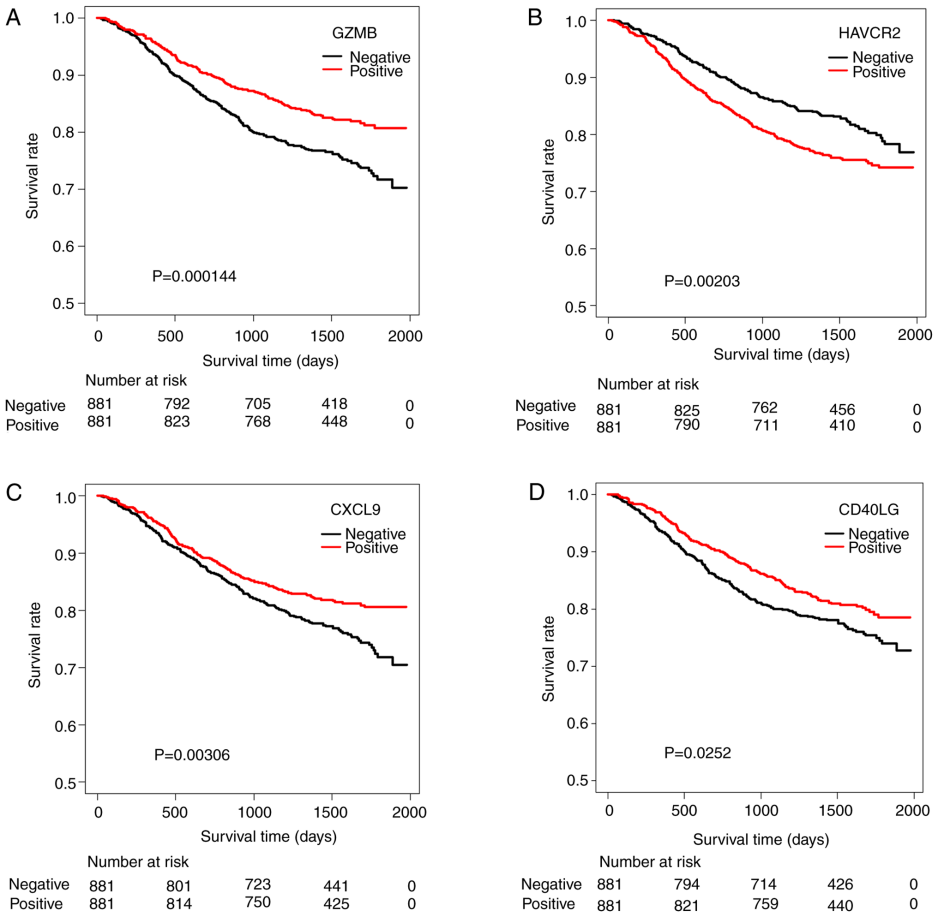


Figure 5. Survival curves of 4 significant prognostic genes identified using the Cox hazards regression model. Overall survival time between patients with positive (higher expression than the median level of an individual gene) and negative expression (lower expression than the median level of an individual gene) was compared by the log-rank test using EZR software. (A) GZMB, (B) HAVCR2, (C) CXCL9 and (D) CD40LG.

dendritic cells in the TME (37), which means that the presence of mature dendritic cells in the tumor site could be a key factor in the prediction of ICB efficacy.

Considering that immunological conditions are varied and complicated in the TME, the status of patients with cancer is volatile and undetermined before the start of treatment. Most likely, immune type group A (PD-L1^{+}CD8^{+}) could be a good candidate to elicit neoantigen-specific T cell reactions and result in an improved prognosis in cancer patients. Efficient combination therapy with chemo- and radiation therapy should be explored for these types of cancer cohorts in the future.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the National Bioscience Database Center repository (accession no. hum0127; https://humandbs.biosciencedbc.jp/en/).

Authors' contributions

RK and YA participated equally in the design of the study and drafting of the manuscript, and were responsible for completing the study. AS, YO, MTe, KUe, TO, SN, YH, NH, YK, YT, HKat, MNi, KT, HKas, MNa and YI were responsible for the clinical work, including the collection of clinical samples. TN, YS, KUr, KO, AI, HM, CM, AK, KW and TA participated in the design of the experiments and performed the genetic analysis. TS contributed to the pathological diagnosis. AN and KM contributed to data analysis and interpretation and confirmed the authenticity of all the raw data. MTa, HKe and KY designed the current study, revised the manuscript critically for important intellectual content and gave final approval of the version to be published by taking responsibility for the content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Shizuoka Cancer Center launched Project HOPE based on multiomics analyses, including WES and GEP. Ethics approval for the HOPE study was obtained from the institutional review board at the Shizuoka Cancer Center (authorization no. 25-33). Written informed consent was obtained from all patients enrolled in the study.

Patient consent for publication

Written informed consent was obtained from all patients for the publication of any associated data and accompanying images.

Competing interests

The authors declare that they have no competing interests.

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