TNFSF9 is associated with favor tumor immune microenvironment in patients with renal cell carcinoma who are treated with the combination therapy of nivolumab and ipilimumab

Bunpei Isoda  
University of Tsukuba

Shuya Kandori  
shuya79@md.tsukuba.ac.jp

Tomokazu Sazuka  
Chiba University

Takahiro Kojima  
Aichi Cancer Center

Satoshi Nitta  
University of Tsukuba

Masanobu Shiga  
University of Tsukuba

Yoshiyuki Nagumo  
University of Tsukuba

Ayumi Fujimoto  
Chiba University

Takayuki Arai  
Chiba University

Hiroaki Sato  
Chiba University

Bryan J. Mathis  
University of Tsukuba Affiliated Hospital

Chia-Ling Wu  
ACT Genomics, Co. Ltd

Yi-Hua Jan  
ACT Genomics, Co. Ltd

Tomohiko Ichikawa
Abstract

Combination therapy of nivolumab and ipilimumab (NIVO + IPI) for metastatic renal cell carcinoma (mRCC) has shown efficacy, but approximately 20% of patients experience disease progression in the early stages of treatment. No useful biomarkers have been reported to date. Therefore, it is desirable to identify biomarkers to predict treatment response in advance. We examined the tumor microenvironment (TME)-related gene expression in mRCC patients treated with NIVO + IPI, between the response and non-response groups, using tumor tissues before administering NIVO + IPI. In TME-related genes, TNFSF9 expression was identified as a candidate for the predictive biomarker. Its expression discriminated between the response and non-response groups with 88.89% sensitivity and 87.50% specificity (AUC = 0.9444). We further analyzed the roles of TNFSF9 in TME, using bioinformatics of The Cancer Genome Atlas (TCGA) cohort. Adaptive immune response was activated in the TNFSF9-high expression tumors. Indeed, T follicular helper cells, plasma B cells, and tumor-infiltrating CD8+ T cells were increased in the tumors, which indicates the promotion of humoral immunity due to enhanced T-B interactions. However, as the number of regulatory T cells (Treg) increased in the tumors, the percentage of dysfunctional T cells also increased. These suggest that not only PD-1 but also CTLA-4 inhibition may have suppressed Treg activation and improved the therapeutic effect in the TNFSF9-high expression tumors. Therefore, TNFSF9 may predict the therapeutic efficacy of NIVO + IPI for mRCC and allow more appropriate patient selection.

Introduction

CheckMate-214 trial has demonstrated the efficacy of combination therapy with nivolumab and ipilimumab (NIVO + IPI) in patients with metastatic renal cell carcinoma (mRCC). Especially, NIVO + IPI brings durable response for some patients. However, approximately 20% of patients experience disease progression in the early stages of NIVO + IPI (1). Therefore, there is a need to identify biomarkers to predict treatment response in advance.

The biomarker analysis of the CheckMate-214 trial investigated genomic and transcriptomic biomarkers and showed the association between these biomarkers and survival outcomes in patients treated with NIVO + IPI (2). PD-L1 expression level was significantly associated with better progression-free survival (PFS) or overall survival (OS) in mRCC patients who were treated with NIVO + IPI. On the other hand, genomic features such as tumor mutation burden (TMB) and tumor indel burden (TIB) did not associate with response to NIVO + IPI. Neither PFS nor OS was significantly associated with the mutation status of the hallmark genes except PBRM1 (2). Moreover, gene expression signatures did not independently predict PFS or OS in these patients. This study suggests that combining multiple gene expression signatures may predict response to NIVO + IPI. However, no effective biomarkers have been identified to predict the therapeutic efficacy of NIVO + IPI for mRCC in clinical practice.

We analyzed the tumor microenvironment (TME)-related genes to predict the responses to NIVO + IPI for mRCC patients, using pretreated primary tumor tissues. TNFSF9 expression was significantly upregulated in the responder group, compared to the non-responder groups. The transcriptomic analysis using The
Cancer Genome Atlas (TCGA)-kidney renal clear cell carcinoma (KIRC) cohort revealed an increase in dysfunctional T cells with regulatory T cells (Treg) in the high TNFSF9 expression tumors, despite the activation of adaptive immune response. It suggests that not only PD-1 but also CTLA-4 inhibition may have suppressed Treg activation and improved the therapeutic effect for the high TNFSF9 expression tumors.

**Materials and Methods**

**Patients**

We included 17 mRCC patients who were treated with NIVO + IPI at the University of Tsukuba or Chiba University hospitals from 2018 to 2020. Patients were divided into response and non-response groups. The responder group included patients with a complete or partial response as the best treatment response and the non-responder group included patients with progressive disease.

**DNA and RNA isolation**

A total of 6 biopsy and 11 surgical specimens were obtained from 17 mRCC patients. For analysis, 5-µm-thick slices were prepared from formalin-fixed paraffin-embedded (FFPE) blocks before total DNA and RNA were extracted from these samples with a RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE (Thermo Fisher Scientific, Waltham, MA, US) according to the manufacturer’s instructions.

**ACTOnco next-generation sequencing**

Extracted genomic DNA was amplified using four pools of primer pairs that target coding exons of analyzed genes. Amplicons were ligated with barcoded adaptors. Quality and quantity of amplified libraries were determined using the fragment analyzer (AATI) and Qubit (Invitrogen, Thermo Fisher Scientific). Barcoded libraries were subsequently conjugated with sequencing beads by emulsion PCR and enriched using an Ion Chef system (Thermo Fisher Scientific) according to Ion PI Hi-Q Chef Kit (Thermo Fisher Scientific) or Ion 540 Kit-Chef protocols (Thermo Fisher Scientific). Sequencing was performed on an Ion Proton or Ion S5 sequencer (Thermo Fisher Scientific).

Raw reads generated by the sequencer were mapped to the hg19 reference genome using the Ion Torrent Suite (version 5.10). Coverage depth was calculated using the Torrent Coverage Analysis plug-in. Single nucleotide variants (SNVs) and short insertions/deletions (INDELs) were identified using the Torrent Variant Caller plug-in (version 5.10). The coverage was down-sampled to 4000. OncoKB (version 20231206) was used to annotate every variant. Variants with coverage ≥ 25, allele frequency ≥ 5% and actionable variants with allele frequency ≥ 2% were retained. This test provides uniform coverage of the targeted regions, enabling target base coverage at 100x ≥ 85% with a mean coverage ≥ 500x. Variants reported in Genome Aggregation database r2.1.1 with > 1% minor allele frequency (MAF) were considered as polymorphisms. An ACT Genomics in-house database was used to determine technical errors. Clinically actionable and biologically significant variants were determined based on the published medical literature.
The copy number variations (CNVs) were predicted as follows: Amplicons with read counts in the lowest 5th percentile of all detectable amplicons and amplicons with a coefficient of variation $\geq 0.3$ were removed. The remaining amplicons were normalized to correct the pool design bias. ONCOCNV was applied for the normalization of total amplicon number, amplicon GC content, amplicon length, and technology-related biases, followed by segmenting the sample with a gene-aware model. This method was also used for establishing the baseline of copy number variations from samples in the ACT Genomics in-house database. (3)

**ACT TME**

ACT TME™ utilizes TaqMan™ OpenArray® (Thermo Fisher Scientific) technology to measure expression signature of 106 immune-related genes involved in antigen presentation, immune checkpoint regulation, immune cell population identification, and other modulators relevant to the tumor microenvironment. In terms of the standard workflow for using TaqMan™ OpenArray® instrument, FFPE RNA was first reverse-transcribed to cDNA by GeneAmp PCR System 9700 (Thermo Fisher Science) before a pre-amplification step to ensure sufficient template for qPCR reaction by a Veriti 96-Well Thermal Cycler (Thermo Fisher Science). Next, the mixture of sample and master mix was loaded onto a QuantStudio 12K Flex OpenArray chip (Thermo Fisher Science), which contained 48 subarrays, using a QuantStudio 12K Flex AccuFill System (Thermo Fisher Science). Finally, the sample-filled OpenArray chip was uploaded to a QuantStudio 12K Flex Real-Time PCR System. (Thermo Fisher Science)

**Use of transcriptomic data from The Cancer Genome Atlas**

Gene expression datasets and clinicopathological data were downloaded from the TCGA-KIRC project (http://cancergenome.nih.gov) in April 2020. The available transcriptomics data from 532 clear cell renal cell carcinoma (ccRCC) specimens whose sample type codes were registered as 01A were used.

**Estimation of stromal and immune cells in tumors**

The levels of infiltrating stromal and immune cells were estimated for each sample based on the gene expression profiles utilizing the “Estimation of STromal and Immune cells in MA lignant Tumours using Expression data” (ESTIMATE) algorithm (https://bioinformatics.mdanderson.org/publicsoftware/estimate/) (4).

**Metascape**

Metascape (v3.5.20240101), an online bioinformatics tool, was employed for gene list enrichment analysis in this study. It facilitates the functional interpretation of gene sets by integrating multiple annotation sources (https://metascape.org/gp/index.html#/main/step1) (5).

**Estimation of tumor-infiltrating immune cells in tumors**

The Tumor IMmune Estimation Resource 2.0 (TIMER2.0) (http://cistrome.org/TIMER/) (6) was used to estimate the population of tumor-infiltrating immune cells.

**Evaluation of T cell signatures in tumors**
Tumor Immune Dysfunction and Exclusion (TIDE) scores were calculated in all samples (http://tide.dfci.harvard.edu/login/) (7). The TIDE algorithm was used to estimate the projected response of each sample to anti-PD-1/PD-L1 and anti-CTLA4 immunotherapy based on gene expression profiles.

**Statistical analyses**

All statistical analyses were performed using R4.3.1 (R Development Core Team, Vienna, Austria), JMP 10 software (SAS Institute, Cary, NC, USA) or GraphPad Prism8 (GraphPad Software, San Diego, CA).

The significance of any differences between groups was assessed by Fisher’s exact test, Wilcoxon rank sum test, or Mann-Whitney U test. Patients were divided into two groups, a low-expression or high-expression group, using the cutoff of median expression values. P-values < 0.05 were considered statistically significant.

**Result**

**Patient Characteristics**

Of 17 total patients, 8 (8 male, no female) were classified into the responders group and 9 (8 male, 1 female) were classified into the non-responders group. Patient characteristics are summarized in Table 1. The median ages of responders and non-responders were 70 years (60–73 years) and 69 years (52–81 years). Regarding IMDC risk, in responders, 7 patients were in the intermediate risk group and 1 patient was in the poor risk group while, in the non-responders, 7 patients were in the intermediate risk group and 2 patients were in the poor risk group. No patient had brain metastases. There were no significant differences in patient characteristics between each group.

**PD-L1, MSI and TMB status**

There were PD-L1 positive tumors (37.5%) in responders and 1 PD-L1 positive tumor (11.1%) in non-responders, but there were no significant differences between groups. (p = 0.2941) None of them showed microsatellite instability-high or tumor mutation burden in our cohort (Table 2).

**Gene mutation status**

In this patient cohort, oncoprint analyses were created by focusing on variants that were presumed to be oncogenic drivers in OncoKB among the genes that were found to be mutated in this study (Fig. 1). The most frequently mutated genes were $VHL$ (47%), $PBRM1$ (41%), and $SETD2$ (24%). In terms of the percentage of these gene mutations, they are slightly more common in $SETD2$, but the rest are comparable to those reported in the past.(8) (9) (10) (11) There were no significant differences in the expression frequencies of these genes between responders and non-responders. (Table 3)
Tumor microenvironment profiling for the immunotherapy biomarker exploration

Almost differentially expressed genes (DEGs) were upregulated in the response group compared to the non-response group. (Fig. 2A) DEGs were defined as genes with a fold change of 2-fold or more and a p-value of 0.05 or less. TNFSF9, IDO1, TIGIT, ICOS, CCL5, ITK, FASLG, CRTAM, HLA-A, GNLY, and INFγ were extracted as DEGs. (Fig. 2B). The most significant difference and fold change was seen in TNFSF9 (tumor necrosis factor superfamily member 9), also known as 4-1BBL or CD137L. TNFSF9 is expressed primarily on antigen-presenting cells and has been shown to function as a potent co-stimulatory molecule. (12) Furthermore, the heatmap for these DEGs shows the expression pattern changes with treatment response. (Fig. 2C)

Also, when ROC analysis is performed for each gene, there are 12 genes with p-values less than 0.05. Of these, 3 have an area under the curve (AUC) greater than 0.8: TNFSF9, TAP1, and CD8A. (Fig. 3A) Interestingly, significant differences in each gene show the smallest p-value for TSFNF9 (p = 0.0010). (Fig. 3B) The AUC is also largest for TNFSF9 (AUC = 0.9444). (Figs. 3A,3C) These results suggest that TSFNF9 is a potential predictive biomarker for the immunotherapy in patients with mRCC.

Characteristics of tumor immune microenvironment in high TNFSF9 expression tumors

To characterize the TIME of high TNFSF9 tumors in KIRC cohort, we calculated the stromal score, immune score, and ESTIMATE score. The ESTIMATE score is a method that estimates the percentage of stromal cells and immune cells in a tumor sample using gene expression signatures. High expression of TNFSF9 was associated with significantly higher Immune scores and higher ESTIMATE scores, but not Stromal scores (Fig. 4A). Therefore, tumors with high TNFSF9 expression were characterized by higher tumor purity and higher immune cell infiltration.

Analysis of DEGs using Metascape revealed that gene pathways related to the adaptive immune responses, response to the bacteria, and NABA MATRISOME (a collection of extracellular matrix-associated proteins and related factors)(13), were upregulated in TNFSF9-high expressing tumors (Fig. 4B). In addition, we analyzed the population of immune cells infiltrating tumor cells using TIMER, finding that B cells, CD8 + T cells, and myeloid dendritic cells were significantly increased in the high-TNFSF9 expression group (Fig. 4C). These findings suggest that tumors with high TNFSF9 expression have more antigen-presenting cells and occur a certain level of acquired immune responses. It is also possible that in the TNFSF9-high expression group, the immune response is better regulated by the interrelationship between the extracellular matrix and immune cells. (14)

Changes in T-cell signatures by TNFSF9 expression

Analysis of T cells by TIDE also showed significantly more CD8+ T cells and significantly higher levels of interferon-gamma in the TNFSF9-high tumors (Figs. 5A and 5B). In addition, there were significantly fewer exclusionary T cells and more dysfunctional T cells in the TNFSF9-high tumors (Figs. 5C and 5D),
suggesting that T cell function may be more impaired in the tumors. However, the association between TNFSF9 and CD274 expression was not significant (Fig. 5E).

TIDE score is calculated using two mechanisms of immune evasion: induction of T-cell dysfunction in tumors with a high infiltration of cytotoxic T cells and inhibition of T-cell infiltration in tumors with few cytotoxic T cells. In general, patients with high TIDE scores are less likely to respond to immune checkpoint inhibitor (ICI) monotherapy (CTLA4 inhibitors or PD-1 inhibitors), and the predominance of high TIDE scores in the TNFSF9-high tumor suggests that ICI monotherapy may be less effective (Figs. 5F and 5G).

**Characteristics of tumor-infiltrating immune cells in high TNFSF9 expression tumors**

Populations of tumor-infiltrating immune cells by TNFSF9 expression were examined using CIBERSORT method (Fig. 6). Plasma B cell, CD8⁺ T cell, T follicular helper (T FH ) cell, regulatory T (Treg) cell, and M1 Macrophage were significantly increased in the TNFSF9-high expression tumor. In contrast, activated mast cell is significantly decreased. The increase of Treg cells may cause dysfunction of T cells in the TNFSF9-high expression tumors (Fig. 5C and Fig. 6). On the other hand, the increase of T FH cells and plasma B cell suggests that T-B interaction leads to humoral immunity for ccRCC in the TNFSF9-high expression tumors (Fig. 6). These findings suggest that both adaptive immune response and dysfunction of T effector cells are promoted in high TNFSF9 tumors.

**Discussion**

The prognosis of mRCC has improved in immunotherapy era. ICIs have emerged as the mainstay and backbone of the treatment strategy for most patients with mRCC. Combination therapy with ICIs: NIVO + IPI or ICI plus tyrosine kinase inhibitor (TKI) combination therapy (IO + TKI) are the first-line treatment options for mRCC in the NCCN guideline (15). Comparing NIVO + IPI to IO-TKI, the duration of response (DOR) is about 2 years for IO-TKI (16) (17), whereas NIVO + IPI has proven a longer DOR in patients with mRCC (18). However, NIVO + IPI has been shown to cause about 20% disease progression in the early stages of treatment. (1) Therefore, it is desirable to find the biomarkers that predict therapeutic response to NIVO + IPI and to identify suitable patients for treatment. In our current study, we found that TNFSF9 expression is most predominantly elevated in the response group of NIVO + IPI. Moreover, its expression discriminated between the response group and non-response group with 88.89% sensitivity and 87.50% specificity (AUC = 0.9444) in our cohort. It suggests that TNFSF9 is a potential biomarker to predict the efficacy of NIVO + IPI for patients with mRCC.

TNFSF9 is expressed not only on antigen-presenting cells but also expressed on non-immune cells. (19) Its receptor, tumor necrosis factor receptor superfamily member 9 (TNFRSF9), is a member of the TNF receptor superfamily and has been identified as a co-stimulatory molecule for T cells. (20) This receptor is expressed as a transmembrane protein on the cell surface and functions to receive and transmit signals to the expressing cells by ligand binding. It has been reported in monocytes to induce proliferation.
(21), differentiation (22), maturation, and production of inflammatory cytokines (23), while inhibiting proliferation through apoptosis in T lymphocytes (24). Therefore, these findings speculate that TNFSF9-TNFRSF9 interaction affects for TIME in mRCC patients who treated with NIVO + IPI.

The relationship between TNFSF9-TNFRSF9 and TIME has been reported in several studies. Cho et al. revealed that in non-small cell lung cancer, reduced levels of TNFRSF9 expression in Tregs improve OS and response to anti-PD-1 antibody immunotherapy. The authors speculate that TNFRSF9 is related to enhanced immunosuppressive activity of Tregs in the tumor. However, they also reported that Treg expression levels did not correlate with response to anti-PD-1 antibody therapy in patients with malignant melanoma, using two independent cohorts. (25) On the other hand, Wu et al. revealed that TNFSF9 expression of the tumor and CD8+ T cell infiltration were negatively correlated in pancreatic cancer. (26) Conversely, $TNFSF9$ expression in RCC was positively associated with CD8+ T cell infiltration in this study. These findings may be attributed to the different roles of $TNFSF9$-TNFRSF9 in the TIME according to cancer type.

The relationship between RCC and TNFSF9 has not been previously reported. A gene pathway analysis using Metascape revealed that the adaptive immune response is promoted in $TNFSF9$-high tumors. Moreover, an analysis using CIBERSORT revealed that both $T_{FH}$ cells and plasma B cells are increased in the tumors. These results suggest that T-B interactions lead to humoral immunity through the promotion of adaptive immune system in $TNFSF9$-high tumors. However, our analysis also revealed that tumor-infiltrating CD8+ T cells, especially dysfunctional CD8+ T cells, are increased in the tumors. Many factors contribute to CD8+ T cell dysfunction, one of which has been shown to involve Tregs (27). CTLA-4, expressed on Tregs, competes with CD28 for binding to CD86/80 on antigen-presenting cells, exerting inhibitory control over CD8+ T cell activation (28). Anergy induction by Tregs leads to CD8+ T cell unresponsiveness, impairing their ability to mount an effective immune response. Furthermore, immunosuppressive factors, such as TGF-β and IL-10, released by Tregs, contribute to the immunosuppression of CD8+ T cells, inhibiting effector cytokine production and fostering an anti-inflammatory milieu (29). Tregs also sequester IL-2, essential for T cell proliferation, limiting its availability for CD8+ T cell expansion (30). Indeed, CIBERSORT analysis showed that Tregs were predominantly increased in $TNFSF9$-expressing tumors. Thus, the increase in Tregs may be the cause of the increase in dysfunctional CD8+ T cells. Moreover, the previous study has demonstrated that TNFSF9 containing extracellular vesicles from cancer cells promote the immunosuppressive activity of Tregs in leukemia (31). Therefore, these findings suggest that TNFSF9 promotes T cell exhaustion via activation of Tregs in the TIME of mRCC.

Nivolumab binds to PD-1 on T cells and blocks its interaction with tumor-expressed PD-L1, thereby inhibiting immune evasion and promoting T cell activation. However, tumor-infiltrating CD8+ T cells and Treg cells express PD-1 at similarly high levels, and administration of anti-PD-1 antibodies may cause inadvertent activation of Tregs and enhancement of immunosuppression (32). To address this, inhibiting receptors that are highly expressed on tumor-infiltrating Tregs can enhance therapeutic efficacy; CTLA-4
is one such receptor. Ipilimumab may play an important role in inhibiting CTLA-4, thereby reducing Treg activation and improving therapeutic efficacy. (33) (34) Collectively, these findings support the rationale that the combination therapy with NIVO + IPI might be effective for mRCC patients with TNFSF9-high tumors.

**Conclusion**

Taken together, our results indicated that TNFSF9 is a potential biomarker to predict the efficacy of NIVO + IPI for patients with mRCC. Moreover, a bioinformatics analysis suggests that TNFSF9 is associated with a favorable TIME for combination therapy with NIVO + IPI in mRCC patients. Additional studies are needed to understand the detailed mechanisms of how TNFSF9 affects the TIME in RCC, especially regarding Treg functions.

**Declarations**

**Funding**

No funding or financial support was received for this study.

**Competing Interests**

*The authors have no relevant financial or non-financial interests to disclose.*

**Author Contributions**

BI reviewed the literature, collected, and analyzed patient data, and prepared the manuscript. SN, MS, AF, TA, and HS collected patient data. YJ, CW analyzed the data. BJM analyzed the data and revised the manuscript. SK, TS, TK, TI, and HN contributed conception and design of the study. The final manuscript was read and approved by all authors.

**Data Availability**

*The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.*

**Ethics approval**

The studies involving human participants were reviewed and approved by Tsukuba University Hospital Institutional Review Board (#H28-104).

**Consent to participate**

*Informed consent was obtained from all individual participants included in the study.*
Acknowledgment

We extend special thanks to ACTmed Co.Ltd. for their coordination between Universities and ACTGenomics Co. Ltd. and support throughout this research.

References

dependent relationship. Science 379:eabp8964. 10.1126/science.abp8964
treatment for advanced renal cell carcinoma (CheckMate 9ER): long-term follow-up results from an
open-label, randomised, phase 3 trial. Lancet Oncol 23:888–898. 10.1016/s1470-2045(22)00290-x
17. Grünwald V, Powles T, Kopyltsov E et al (2023) Survival by Depth of Response and Efficacy by
International Metastatic Renal Cell Carcinoma Database Consortium Subgroup with Lenvatinib Plus
Pembrolizumab Versus Sunitinib in Advanced Renal Cell Carcinoma: Analysis of the Phase 3
Randomized CLEAR Study. Eur Urol Oncol 6:437–446. 10.1016/j.euo.2023.01.010
10.4110/in.2009.9.3.84
20. Shao Z, Schwarz H (2011) CD137 ligand, a member of the tumor necrosis factor family, regulates
Blood 94:3161–3168
independent of CD95. Immunology 98:42–46. 10.1046/j.1365-2567.1999.00851.x
marker of tumor-infiltrating regulatory T-cell enabling clinical outcome prediction in lung cancer.
responses. Nat Rev Immunol 11:852–863. 10.1038/nri3108
Immunologic self-tolerance maintained by CD25+ CD4+ naturally anergic and suppressive T cells:


Tables

Table 1 to 3 are available in the Supplementary Files section.

Figures

Figure 1: OncoPrint of gene mutations between responders and non-responders
Figure 1

Oncoprint of gene variants in primary tumors between responders and non-responders in our cohort. Red indicates a missense variant, blue indicates a frameshift variant, green indicates a gain of stop codon, yellow indicates copy number alteration, and light blue indicates an in-frame deletion.

Figure 2: Differentially expressed genes (DEGs) in primary tumors between responders and non-responders

A) A volcano plot showing the distribution of the DEGs between Responders and Non-responders. B) A list of the significant DEGs between Responders and Non-responders. C) A heatmap of the 11 significant DEGs. Shades of blue and red correlate with increasing or decreasing Z scores (from -2 to 2), with white representing a Z score of 0.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Immune subpopulation</th>
<th>P-value</th>
<th>Fold Change</th>
<th>Elevated</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFSP5</td>
<td>Immune Checkpoint</td>
<td>0.00163961</td>
<td>4.26289</td>
<td>Responder</td>
</tr>
<tr>
<td>ILOI</td>
<td>Immune Checkpoint</td>
<td>0.001578</td>
<td>2.45453</td>
<td>Responder</td>
</tr>
<tr>
<td>TGF</td>
<td>Immune Checkpoint</td>
<td>0.0281069</td>
<td>2.85568</td>
<td>Responder</td>
</tr>
<tr>
<td>IKG</td>
<td>Immune Checkpoint</td>
<td>0.045546</td>
<td>2.86074</td>
<td>Responder</td>
</tr>
<tr>
<td>CGL5</td>
<td>Activated CD4 T cell</td>
<td>0.034548</td>
<td>2.39538</td>
<td>Responder</td>
</tr>
<tr>
<td>FIK</td>
<td>Activated CD4 T cell</td>
<td>0.0399679</td>
<td>2.44416</td>
<td>Responder</td>
</tr>
<tr>
<td>FASLG</td>
<td>Natural killer cell</td>
<td>0.0235268</td>
<td>2.57054</td>
<td>Responder</td>
</tr>
<tr>
<td>CTRAM</td>
<td>Natural killer cell</td>
<td>0.0238298</td>
<td>2.60554</td>
<td>Responder</td>
</tr>
<tr>
<td>HLA-A</td>
<td>IdmC</td>
<td>0.0432763</td>
<td>2.31468</td>
<td>Responder</td>
</tr>
<tr>
<td>GILY</td>
<td>Activated CD8 T cell</td>
<td>0.0433385</td>
<td>2.77134</td>
<td>Responder</td>
</tr>
<tr>
<td>PNG</td>
<td>Catalytic Activity</td>
<td>0.047294</td>
<td>3.84982</td>
<td>Responder</td>
</tr>
</tbody>
</table>

Figure 2

The differentially expressed genes (DEGs) in primary tumors between Responders and Non-responders. (A) A volcano plot showing the distribution of the DEGs between Responders and Non-responders. (B) A list of the significant DEGs between Responders and Non-responders. (C) A heatmap of the 11 significant DEGs. Shades of blue and red correlate with increasing or decreasing Z scores (from -2 to 2), with white representing a Z score of 0.
Figure 3: Receiver Operating Characteristics (ROC) analysis

(A) The List of potential biomarker genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Immune Subpopulation</th>
<th>p-value</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFSF9</td>
<td>Immune Checkpoint</td>
<td>0.001</td>
<td>0.944</td>
</tr>
<tr>
<td>CAF1G</td>
<td>Activated dendritic cell</td>
<td>0.011</td>
<td>0.726</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Tumor Infiltration Chemokines</td>
<td>0.015</td>
<td>0.556</td>
</tr>
<tr>
<td>SIRPI</td>
<td>Immune Checkpoint</td>
<td>0.021</td>
<td>0.421</td>
</tr>
<tr>
<td>BST2</td>
<td>Natural killer cell</td>
<td>0.027</td>
<td>0.421</td>
</tr>
<tr>
<td>GNLY</td>
<td>Activated CD8 T cell</td>
<td>0.037</td>
<td>0.528</td>
</tr>
<tr>
<td>IDO1</td>
<td>Immune Checkpoint</td>
<td>0.037</td>
<td>0.664</td>
</tr>
<tr>
<td>CCL5</td>
<td>Activated CD4 T cell</td>
<td>0.046</td>
<td>0.500</td>
</tr>
<tr>
<td>CD95</td>
<td>Effector memory CD8 T cell</td>
<td>0.046</td>
<td>0.653</td>
</tr>
<tr>
<td>CDB8A</td>
<td>Activated CD8 T cell</td>
<td>0.046</td>
<td>0.519</td>
</tr>
<tr>
<td>FASLG</td>
<td>Natural killer cell</td>
<td>0.046</td>
<td>0.684</td>
</tr>
<tr>
<td>TAP1</td>
<td>IdMC</td>
<td>0.046</td>
<td>0.861</td>
</tr>
</tbody>
</table>

(B) The gene expression levels between Responders and Non-responders.

(C) ROC curves of TNFSF9, showing sensitivity and specificity of the analysis regarding TNFSF9 prediction of NIVO + IPI response. AUC = area under curve.

Figure 3

Receiver Operating Characteristics (ROC) analysis. (A) The List of potential biomarker genes. (B) The gene expression levels between Responders and Non-responders. (C) ROC curves of TNFSF9, showing sensitivity and specificity of the analysis regarding TNFSF9 prediction of NIVO + IPI response. AUC = area under curve.
Figure 4: The evaluation of tumor immune microenvironment using ESTIMATE between low and high-TNFSF9 tumors

(A) The stromal, immune, and ESTIMATE score between high and low TNFSF9 tumors were evaluated by the ESTIMATE algorithm. (B) The difference of Gene Ontology biological process categories between high and low TNFSF9 tumors. (C) The proportion of tumor-infiltrating immune cells between high and low TNFSF9 were evaluated by the TIEMR algorithm.

The evaluation of tumor immune microenvironment between low and high TNFSF9 tumors. (A) The stromal, immune, and ESTIMATE score between high and low TNFSF9 tumors were evaluated by the ESTIMATE algorithm. (B) The difference of Gene Ontology biological process categories between high and low TNFSF9 tumors. (C) The proportion of tumor-infiltrating immune cells between high and low TNFSF9 were evaluated by the TIEMR algorithm.
Figure 5: Differences in T cell signatures between low- and high-
TNFSF9 tumors (TIDE)

* Mann Whitney test

A) CD8+ T cell

B) IFN-γ

C) Dysfunction T cell

D) Exclusion T cell

E) Expression status of CD274 (PD-L1)

F) TIDE score

G) Response rate to immune checkpoint inhibitor monotherapy as predicted by TIDE score.

Figure 5

The evaluation of T cell signatures using TIDE between low and high TNFSF9 tumors (A) CD8+ T cell (B) Interferon gamma (C) Dysfunction T cell (D) Exclusion T cell (E) Expression status of CD274 (PD-L1) (F) TIDE score (G) Response rate to immune checkpoint inhibitor monotherapy as predicted by TIDE score.
Figure 6: The proportion of tumor-infiltrating immune cells between low- and high-\textit{TNFSF9} tumors (CIBERSORT-ABS).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Slide1.png
- Slide2.png
- Slide4.png