IGSF6 is a novel biomarker to evaluate immune infiltration in mismatch repair-proficient colorectal cancer

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Research Article

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Abstract

**Background:** Immunotherapy has dramatically changed the landscape of treatment for colorectal cancer (CRC), but there is lack of effective predictive biomarker, especially for tumors with mismatch repair (MMR) proficiency. Immune response relies to cell surface receptors and their interactions, such as cell-cell recognition, binding and adhesion. However, the function of immunoglobulin superfamily (IGSF) genes in tumor immune microenvironment remains uncharacterized.

**Methods:** This study quantified the immune using the gene expression matrix obtained from the public database. Also the associations between IGSF6 gene expression and immune cell infiltration were assessed. The expression levels of IGSF6, CD8+ and CD4+ T cells in cancer tissues from CRC patients were evaluated.

**Results:** IGSF6 was more highly expressed in CRC tumor tissues than corresponding adjacent normal tissues. And IGSF6 was significantly correlated with immune cell infiltration in MMR-proficient patients. Remarkably, MMR-proficient patients with high IGSF6 expression showed more sensitive to immunotherapy and chemotherapy than those with low IGSF6 expression.

**Conclusions:** In summary, IGSF6 could be a novel biomarker to evaluate immune infiltration and predict therapeutic effect for MMR-proficient CRC.

Introduction

Immunotherapy is demonstrated as a promising strategy for many types of cancers with long-term durable responses, such as for breast cancer [1], lung cancer and melanoma [2, 3]. But there are still lots of challenges in immunotherapy for colorectal cancer (CRC) patients. CRC are classified into MMR-deficient (microsatellite instability) and MMR-proficient (microsatellite stability) subtypes. Programmed death 1 (PD-1) blockade is verified as breakthrough therapy for MMR-deficient CRC, but less effective in MMR-proficient CRC [4–6]. MMR-deficient tumors are characterized by a high tumor mutational burden (TMB) and high infiltration of activated CD8+ cytotoxic T lymphocytes (CTL) and activated Th1 cells with IFN-γ production [7]. These features enable MMR-deficient tumor to be a good target for immunotherapy [8]. On the contrast, MMR-proficient CRC has been long thought to have an inactive response to immune checkpoint inhibitors. The lack of immune infiltration and low TMB in MMR-proficient CRC decrease the potential of obtaining benefits from immunotherapy, which is defined as an “immune resistant” phenotype [9]. Interestingly, about 45% of MMR-proficient tumors had a high immunoscore, which means that some of MMR-proficient tumors may be sensitive to immunotherapy [10]. Consistently, a previous clinical study reported that 27% (4/15) of MMR-proficient patients had pathological responses when treating with ipilimumab plus nivolumab, indicating that immunotherapy may be effectivity in some of MMR-proficient patients [11]. Furthermore, immune hot tumors with extensive immune infiltration also had better responses to chemotherapy [12, 13]. However, there is currently insufficient information to routinely utilise predictive biomarkers for MMR-proficient patients. As more than 90% CRC are MMR-proficient, it is an urgent clinical need to identify effective immune checkpoints for MMR-proficient patients to predict sensibility of immunotherapy.

Increasing evidence show that the tumor immune contexture, including spatial organization and density, directly influence the clinical outcome of cancer [14–17]. The immunoglobulin superfamily (IGSF) consists of the immunoglobulins (IG), T cell receptors (TR) and proteins that have the common feature of having at least one Ig-like domain [18]. It has been reported that IGSF genes are frequently overexpressed in several cancer types and plays a role in promoting cancer cell growth in the progression of cancer, such as thyroid cancer [19] and prostate cancer [20]. Recently, a study reported that IGSF protein signatures are associated with distinct tumor immunophenotypes and clinical outcome [21]. However, it is unclear whether IGSF genes could be used as biomarkers in CRC. In this study, we evaluated a group of IGSF genes in CRC and found IGSF6 may be a novel prognostic biomarker for MMR-proficient CRC. As far as we know, the function of IGSF6 in cancer has not yet to be clarified. We identified IGSF6 was upregulated in CRC tissues, which was correlated with the immune checkpoint genes and immune cell infiltration. Moreover, we demonstrated overexpression of IGSF6 was associated with a high density of CD8+ T cell and CD4+ T cell tumor-infiltrating lymphocytes. Importantly, MMR-proficient tumors with high IGSF6 expression showed a better response to immunotherapy and chemotherapy.
Materials And Methods

Study design and patient selection

Two public cohorts, The Cancer Genome Atlas (TCGA) CRC cohort and GSE39582 dataset from the Gene Expression Omnibus (GEO) database, with gene expression data derived from fresh-frozen CRC samples were evaluated retrospectively. A total of 6 MMR-proficient CRC patients treated by immune checkpoint inhibitors (ICI) plus chemotherapy and 21 MMR-proficient CRC patients underwent neoadjuvant chemotherapy at the Sixth Affiliated Hospital of Sun Yat-Sen University (Guangzhou, China), were retrospectively studied. Patients with infectious diseases, autoimmune diseases, or multiple primary cancers were excluded. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of the Sixth Affiliated Hospital, Sun Yat-sen University (approval number: 2021ZSLYEC-099).

Immunofluorescence

Sections of 5 µm thickness were obtained from CRC tumors. Deparaffinization of slides was done with xylene followed by rehydration in histological-grade ethanol and fixation with 3% hydrogen peroxide in methanol, before antigen retrieval using pH 6.0 citrate buffer or pH 9.0 EDTA buffer. Then the slides were incubated with the primary antibody overnight at 4°C. Slides were scanned and digitalized with and visualized with CASEVIEWER software.

Immunohistochemistry Staining

Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) staining were performed using standard immunoperoxidase staining on CRC tissue sections of 5 µm thickness from resected tumors. Sections were stained against anti-IGSF6 (SANTACRUZ, sc-377053, 1:200), anti-CD4(ZSGB-Bio, ZA-0519), anti-CD8(ZSGB-Bio, ZA-0508). Paraffin sections were deparaffinized with xylene in the stainer and then underwent heat-mediated antigen retrieval with pH 9.0 EDTA buffer. Then the slides were incubated with the primary antibody overnight at 4°C, and the sections were stained with diaminobenzidine. Sections were counterstained with hematoxylin, dehydrated and mounted with coverslips. Slides were scanned and digitalized with the TEKSQRAY image analysis system and visualized with ImageViewerG software. A board-certified pathologist evaluated the staining digitally to ensure the appropriate quality of tumor tissue.

Evaluation Of Immunohistochemical And Immunofluorescence Analysis

Immunoreactivity for IHC and immunofluorescence staining was evaluated by a semiquantitative method, as described previously. Each TMA spot was assigned an intensity score from 1 to 4 (1, 2, 3, or 4) by two trained researchers. The percentage of positive tumor cells divided by the total number of tumor cells was assigned using 25% increments (25%, 50%, 75%, and 100%). IHC and immunofluorescence scores were determined by the intensity score and the proportion of area positively stained tumor cells. A final score was determined as the average of two cores from the same representative tumor area.

Statistical analysis

All statistical analyses were accomplished by R software V.4.2.1 (http://www.r-project.org) and Graphpad Prism 9 software. Data were presented as the mean ± SD, unless otherwise stated. Statistical significance between two groups was evaluated by two-tailed Student's t-test. Statistical significance was considered at p < 0.05.

Results

IGSF6 is highly expressed in CRC tumor tissues and could be a novel biomarker to evaluate immune infiltration.
To characterize the potential function of IGSF genes in CRC, we first investigated the expression of IGSF genes in CRC tumor tissue and adjuvant tissue from Gene Expression Profiling Interactive Analysis (GEPIA, cancer-pku.cn). Interestingly, only IGSF6 was significant up-regulated in both colon and rectal tumor versus normal tissue (Fig. 1A). To confirm the findings in public database, we detected IGSF6 expression in 16 pairs of CRC tissues and adjacent normal colorectum tissues. Unexpectedly, IGSF6 levels are highly expressed in tumor tissues as compared with adjacent normal tissues (p < 0.0001, Fig. 1B-C). Since IGSF genes play a central role in cell-cell recognition as cell surface receptors, these results suggest that IGSF6 may be a neoantigen generated during carcinogenesis and involving in immune infiltration.

Tumor immune microenvironments are important factors to effect immunotherapy in clinic, but currently lock of effective biomarker to evaluate immune infiltration and the effective of immunotherapy. To evaluate IGSF6 can be used as a biomarker for immune infiltration, we assessed the association between IGSF6 expression and tumor-infiltrating lymphocytes in TCGA datasets. The results showed that the expression of IGSF6 was positive correlated with tumor-infiltrating lymphocytes in various cancers, especially for CRC (Fig. 1D). Furthermore, only IGSF6, among IGSF family genes, showed a strong positive correlation with tumor-infiltrating lymphocytes in CRC patients, including MMR-deficient and MMR-proficient tumors (Fig. 1E). Interestingly, a strong positive correlation between IGSF6 expression and tumor-infiltrating lymphocytes can be found in MMR-proficient tumors, indicating that IGSF6 could be an immunotherapy biomarker for MMR-proficient CRC patients (Fig. 1F). What's attractive, IGSF6 was strongly positive correlated with immune checkpoints in MMR-proficient CRC, such as PD-1, PD-L1, CTLA-4, LAG3 and TIGIT (Fig. 1G-H). Moreover, expression of IGSF6 correlated with tumor-infiltrating lymphocytes were confirmed in GSE39582 dataset (Supplementary Fig. 1), which supported that IGSF6 could be a novel biomarker to improve clinical applications of current immunotherapies.

MMR-deficient tumors which have highly tumor mutational burden (TMB), for accumulation of insertions–deletions (indels) which given rise to more neoantigens, are more beneficial from immunotherapy [22–26]. Therefore, we evaluated the association between IGSF6 expression and TMB in CRC, and found a positive correlation between IGSF6 genes and TMB (Fig. 1I).

Furthermore, we investigated the relationship between IGSF6 expression and the tumor immune microenvironment. We calculated single-sample gene set enrichment analysis (ssGSEA) scores for patients using known CRC immune signatures. Given that MMR-deficient tumors have a distinct immunologic profile, we separated them into their own group and performed unsupervised hierarchical clustering on the MMR-proficient tumors (Fig. 1J). We found high IGSF6 expression tumors were associated with extensive immune infiltration, which also be confirmed in another CRC dataset (Fig. 1K).

### High Igsf6 Expression Is Associated Benefit From Immunotherapy And Chemotherapy

As IGSF6 expression was associated with immune infiltration, we next investigated whether IGSF6 could be used as a biomarker to predict therapeutic effect for CRC. A total of 6 MMR-proficient CRC patients treated with immunotherapy were studied (Table 1). After systematic ICI treatment, the patients received staged or simultaneous complete surgical resection for primary tumor. A thorough pathological examination of the resected tumors before and after ICI was conducted. Immuno-sensitive MMR-proficient CRC showed a sharp tumor burden reduction, massive necrosis tissue and lymphocytes infiltration after ICI treatment, such as Case 1 (Fig. 2A, 2H). On the contrary, there was no response or even bad response in immuno-resistance tumors, such as Case 2 (Fig. 2A, 2I). We next detected IGSF6 expression, CD4+ T cell and CD8+ T cell from pathological tumor specimens with IHC. Interestingly, high IGSF6 expression were observed in resected specimens in immuno-sensitive tumors, while with high CD4+ and CD8+ T cell infiltration (Fig. 2A). As expected, IGSF6 levels are more highly expressed in immuno-sensitive tumors than immuno-resistance tumors (Fig. 2B). To further clarify whether the IGSF6 could predict benefit in CRC patients, we focused on the clinical outcome in the IGSF6high and IGSF6low groups. The OS of CRC patients with IGSF6 high expression was better than those with IGSF6 low expression in TCGA cohorts, especially for MMR-proficient patients (Fig. 2C). The same tendency can also be found in GSE39582 cohorts (Fig. 2D).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patient 1</th>
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<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
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<td>Rectum</td>
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<td>Rectum</td>
<td>Colon</td>
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<td>STAGE</td>
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<td>T4aN1aM0</td>
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<tr>
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<td>FOLFOX + radiotherapy (PR)</td>
<td>FOLFOX (PR)</td>
<td>none</td>
<td>FOLFOX (PD)</td>
<td>XELOX (PD)</td>
</tr>
<tr>
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<td></td>
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<td>FOLFOX + sintilimab</td>
<td>FOLFOX + sintilimab</td>
<td>sintilimab + FOLFOX</td>
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<td>nivolumab</td>
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<td>PR</td>
<td>PD</td>
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<tr>
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<td>PR</td>
<td>PD</td>
<td>ξ</td>
<td>ξ</td>
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<tr>
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<td>Dixon</td>
<td>ξ</td>
<td>ξ</td>
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<td>Pathological response</td>
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<tr>
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<td>PD</td>
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<tr>
<td>TRG score (NCCN Guidelines)</td>
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<td>1</td>
<td>1</td>
<td>2</td>
<td>ξ</td>
<td>ξ</td>
</tr>
</tbody>
</table>

¶ Assessed by the Response Evaluation Criteria in Solid Tumors 1.1 criteria.

ξ Colectomy was conducted before ICI combined treatment.

FOLFOX, fluorouracil + oxaliplatin; XELOX, capecitabine + oxaliplatin; ICI, immune checkpoint inhibitor; pMMR, mismatch repair (MMR) proficient; dMMR, mismatch-repair (MMR) deficient; PD, progressive disease; PR, partial response.

Chemotherapy remains a relatively common and effective treatment for most MMR-proficient CRC patients. To further explore the relationship between IGSF6 and patients’ response to chemotherapy, we collected the fresh tissues of chemosensitive and chemoresistance MMR-proficient CRC patients before treatment (Table 2). As expected, IGSF6 levels are more highly expressed in tumor tissues from chemosensitive patients than from chemoresistance patients (Figs. 3A-B). There were more tumor-infiltrating immune cells in tumor tissues of the IGSF6\textsuperscript{High} group than in those of the IGSF6\textsuperscript{Low} group, indicating that MMR-
proficient CRC tissues with high levels of IGSF6 had a tumor microenvironment with an activated adaptive immune phenotype (Figs. 3A-B).

### Table 2
Patient Characteristics of 21 MMR-proficient CRC patients treat with chemotherapy.

<table>
<thead>
<tr>
<th>TRG</th>
<th>Treatment</th>
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<td>T4aN2aM0</td>
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<td>T3N0M0</td>
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<tr>
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<td>FOLFOX</td>
<td>T4aN2aM1</td>
<td>Rectum</td>
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<tr>
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<td>FOLFOX</td>
<td>T3N2aM0</td>
<td>Rectum</td>
</tr>
<tr>
<td>0</td>
<td>UNKNOWED</td>
<td>T4aN2bM0</td>
<td>Rectum</td>
</tr>
<tr>
<td>0</td>
<td>Avastin + FOLFOXIRI</td>
<td>T3N2aM1</td>
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<td>FOLFOXIRI</td>
<td>T3N1aM0</td>
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<td>3</td>
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<td>Rectum</td>
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<tr>
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<tr>
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</table>

FOLFOX, fluorouracil + oxaliplatin; XELOX, capecitabine + oxaliplatin; FOLFOXIRI, fluorouracil + oxaliplatin + Irinotecan.

### Discussion

CRC is one of the most common cancer types in the world due to its high prevalence\(^{27}\). Immunotherapy has shown as an effectively therapeutic strategy for many types of cancers, but its clinical application in CRC only for MMR-deficient patients. A large amount of MMR-proficient tumors had shown a high proportion of inter-tumoral tumor-infiltrating lymphocytes as “hot tumors”, which may be sensitive to immunotherapy\(^{28,29}\). Biomarkers, including CEA, CA199, and CA125, in clinical practice are significance for tumor cell proliferation and tumor recurrence. But lack of effectively biomarker which can predict immune infiltration. Therefore, it is critical for identifying novel predictive markers for MMR-proficient tumors who may achieve benefits from immunotherapy.

Recent studies point out the importance of the tumor immune contexture for the prognosis of patients with CRC\(^{30–33}\). Immunotherapy targeting immune checkpoints (such as PD1/PD-L1) has become an approved treatment option for patients
with CRC with mismatch repair deficiency or high microsatellite instability [34]. Interestingly, about 45% of MMR-proficient and 65% of MMR-deficient CRC had a high immunoscore, while 55% of MMR-proficient and 35% of MMR-deficient CRC had a low immunoscore, suggesting that some of the MMR-deficient tumors are unable to mount an antitumor response while the reverse may apply for some of the MMR-proficient population [35]. Indeed, the 27% pathological response in MMR-proficient early-stage CRC treated with neoadjuvant ipilimumab plus nivolumab provide further support that MMR-proficient CRC is not an immune desert and can be targeted with immunotherapy [36]. Apart from MMR-deficiency and MMR-proficiency, we speculate that IGSF6 gene is another potential biomarker for responsiveness to immunotherapy due to their significant association with TMB, which is an effective indicator for response prediction to ICI. In our study, IGSF6 may be a new biomarker which may promote the expression of immune cells, such as CD8 + T cells associated with improved survival of patients with MMR-proficient CRC [37, 38], contributing to a better immune function. The complex interactions between tumor and immune cells play out in the TME and dictate clinical outcomes, including among patients with CRC. No data are available on CRC, but in several tumor types an increased T cell infiltration (TCI) has been shown to increase the probability of response to ICI [39, 40].

A number of inhibitory immunoreceptors have been identified and studied in cancer in past decades, including but not limited to PD-1, PD-L1, CTLA-4, LAG3, HAVCR2, TIGIT, CD69 and CD40. They are viewed as “immune checkpoints” referring to molecules that act as gatekeepers of immune responses. In the evolutionary process, immune checkpoints have co-evolved with stimulatory immunoreceptors and appear as early as in fish [41]. These receptors often use monoyrosine signaling motifs, such as immunoreceptor tyrosine-based inhibitory motif (ITIM) and immunoreceptor tyrosine-based switch motif (ITSM), to deliver inhibitory signals. As surface molecules, their activity can be easily inhibited by blocking antibodies that prevent ligand-receptor engagement. The most successful immune checkpoint blockade therapy, one of anticancer immunotherapies, is anti-PD-1/PD-L1 therapy that has been approved to treat a wide variety of cancer types, such as blood, skin, lung, liver, bladder and kidney cancers [42]. Immune checkpoint blockade therapy often leads to more durable response than chemo or targeted therapies, perhaps reflecting the memory feature of the immune system. But as clinical data accumulates all over the world, drawbacks and side effects have begun to be revealed. The major bottleneck of immune checkpoint blockade therapy is its low response rate in most cancers, with a range of 10–30% [42]. For some major cancer types such as MMR-proficient CRC, anti-PD-1/PD-L1 therapy shows nearly no effect [43]. In this study, we identified a crucial involvement of IGSF6 in CRC promotion immune cells. IGSF6 expression was upregulated in CRC tissues and high expression of IGSF6 correlated with an active immune microenvironment and a favorable prognosis and furthermore it may facilitate immune mediated tumor killing. IGSF6 could be a new biomarker to design next generation therapies and to improve clinical protocols of current immunotherapies. But this should be validated in further study before its clinical application.

Here, we elucidated that IGSF6 expression promoted more immune cells in CRC, so as to facilitate immune mediated tumor killing. IGSF6 expression was an independent predictor of better prognosis, and these results may provide insight into effective strategies for therapy in MMR-proficient CRC, which enables IGSF6 to be a novel prognostic biomarker and target of immunotherapies.

**Declarations**

**Author Contributions:** Yu-cheng Xu, Zhao-liang Yu, Xiao-chuan Chen and Min-er Zhong contributed to study concept and design, acquisition, analysis, interpretation of data and drafting of the manuscript. Yu-fan Liang and Jing-rong Weng contributed to data collections and manuscript review. Dan-dong Luo and Yi-ran Bie contributed to study concept and design, analysis and interpretation of data and critical revision of the manuscript for important intellectual content. Xi Chen, Jia-wei Cai, Yu-ming Rong and Yi-feng Zou supervised the study. All authors read and approved the final manuscript.

**Ethics approval:** This study was approved by the ethics committees of Sixth Affiliated Hospital of Sun Yat-sen University.

**Consent for publication:** All authors approve to publish this paper.

**Data availability:** All data that supporting the findings of this study are available from the corresponding author upon request.
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**Conflict of Interest Statement:** All authors declare no conflict of interests.

**Acknowledgments:** Not applicable.

**References**


Figures

Figure 1

IGSF6 is highly expressed in CRC tumor tissues and could be a novel biomarker to evaluate immune infiltration. (A) Expression of IGSF from the GEPIA database. (B) Representative immunofluorescence images of IGSF6 expression in CRC and adjacent normal tissues from the patient. (C) Quantification of the score for IGSF6 level in tumor tissue versus adjacent tissue assessed by immunofluorescence assay. * p < 0.05, *** p < 0.001. ** p < 0.0001. p values were determined by paired-t test. (D) Correlation between immune cell infiltration and IGSF6 in pan-cancer. * p < 0.05, ** p < 0.01, *** p < 0.001. (E) Correlation between immune...
cell infiltration and IGSF in CRC patients from TCGA database, n=383. (F) Correlation between immune cell infiltration and IGSF in MMR-proficient CRC patients, n=249. (G, H) Correlation between immune check points and IGSF6 expression in MMR-proficient patients by Spearman's correlation coefficient. n=249. (I) Correlation between TMB and IGSF6 expression in CRC patients by Spearman's correlation coefficient, n=359. (J, K) Unsupervised hierarchical clustering of colorectal tumors using ssGSEA scores for immune signatures identifies increasing levels of immune infiltrates. J from TCGA database, n=367, K from GSE39582 database, n=519.

Figure 2

High IGSF6 expression is benefit from immunotherapy. (A) Radiological and pathological response to FOLFOX plus sintilimab in a patient with stage T3N0M0 (Case 1) and FOLFOX plus sintilimab in a patient with stage T3N1M0 (Case 2). Radiographic imaging shows the tumor in rectum (, ) at initial diagnoses. A notable tumor regression could be seen in primary tumor from Case 1 ( ). But there was no response in Case 2 after ICI ( ). Primary tumor was observed using colonoscopy (, ) at initial diagnosis and after ICI treatment (, ). H&E staining shows primary tumor at initial diagnosis (, ) and pathological response after ICI treatment (VI, ). Fibrosis and an infiltration with viable density of many lymphocytes (VI, arrowheads) can be found, which cannot be found in case 2 ( ). IHC staining showed CD4+ T cells, CD8+ T cells and IGSF6 expression with pretreatment
tumor samples (, , ) and posttreatment tumor tissues (, , ). (B) Quantification of the score for CD4+ T cells, CD8+ T cells and IGSF6 staining before treatment in CRC tissue from immunotherapy sensitive versus immunotherapy resistance assessed by IHC assay, n=6. (C, D) OS curve of patients with high IGSF6 and low IGSF6 group in TCGA database (C) and GSE39582 database (D).

Figure 3

High IGSF6 expression is benefit from chemotherapy. (A) Representative micrographs of CD4+ T cells, CD8+ T cells and IGSF6 staining from endoscopic pathological tumor specimens before patients underwent chemotherapy, n=21. (B) Quantification of the score for CD4+ T cells, CD8+ T cells and IGSF6 staining from chemosensitive CRC versus chemoresistance CRC by IHC assay, n=21. pMMR, mismatch repair (MMR) proficiency; dMMR, mismatch repair (MMR) deficient. * p < 0.05, *** p < 0.001, ****p<0.0001.

Supplementary Files

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