Received: 22 November 2021 | Revised: 9 October 2022 | Accepted: 14 October 2022

DOI: 10.1111/cas.15716

Cancer Science WILEY

ORIGINAL ARTICLE

Association of *KMT2C/D* loss-of-function variants with response to immune checkpoint blockades in colorectal cancer

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Abstract

Immune checkpoint inhibitors (ICIs) have become important treatment strategies, yet responses vary among patients and predictive biomarkers are urgently needed. Mutations in *KMT2C* and *KMT2D* lead to increased levels of genomic instability. Therefore, we aimed to examine whether *KMT2C/D* mutations might be a predictor of immunotherapeutic efficacy. Here, we investigated the associations of *KMT2C/D* loss-of-function (LOF) variants with tumor mutation burden (TMB), MSI-H, PD-L1 expression, the levels of tumor-infiltrating leukocytes (TILs), and clinical response to ICIs. It was found that *KMT2C/D* LOF variants were associated with higher TMB. Compared with the non-LOF group, the proportion of patients with MSI-H tumors was larger in the LOF group. PD-L1 expression was higher in the LOF group only for colorectal cancer in both the Chinese and The Cancer Genome Atlas cohorts. Importantly, *KMT2C/D* LOF variants were associated with decreased regulatory T cells and increased levels of $CD8^+$ T cells, activated NK cells, M1 macrophages, and M2 macrophages in colorectal cancer. However, there was no significant association between *KMT2C/D* LOF

Abbreviations: ARID1A, AT-rich interactive domain-containing protein 1A; DDR, DNA damage response; ICI, immune checkpoint inhibitor; LOF, loss-of-function; MLL, mixed-lineage leukemia; MMR, mismatch repair; MSI-H, microsatellite instability-high; MSS, microsatellite stable; NGS, next-generation sequencing; NK, natural killer; OS, overall survival; POLD1, DNA polymerase delta 1; POLE, DNA polymerase epsilon; TILs, tumor-infiltrating lymphocytes; TMB, tumor mutation burden; Treg, regulatory T cell; Trr, trithorax-related; Trx, trithorax.

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Funding information
Applied Technology Research and Development Program of Heilongjiang Province, Grant/Award Number: GA19C002; National Natural Science Foundation of China, Grant/Award Number: 81872427, 82102988 and 82173233

and TILs levels in other cancer types. Consistently, the results showed that *KMT2C/D* LOF variants were associated with prolonged overall survival only in colorectal cancer ($p = 0.0485$). We also presented that patients with *KMT2C/D* LOF mutations exhibited a better clinical response to anti-PD-1 therapy in a Chinese colorectal cancer cohort ($p = 0.002$). Taken together, these results suggested that *KMT2C/D* LOF variants could be a useful predictor for ICIs efficacy in colorectal cancer. In addition, the predictive value of *KMT2C/D* LOF variants was consistent with their association with TILs levels.

KEYWORDS
immune checkpoint inhibitors, *KMT2C/D* LOF, MSI-H, tumor mutation burden, tumor-infiltrating lymphocytes

1 | INTRODUCTION

Immune checkpoint blockades with antibodies targeting PD-1/PD-L1 and CTLA-4 have changed the therapeutic landscape for many types of cancer. However, the majority of patients do not respond well to ICI therapy.[1-4] Therefore, novel predictive biomarkers are urgently needed to identify patients who can benefit from immunotherapy.

Studies have shown that alterations in genes involved in DNA repair and DDR can act as predictive biomarkers for response to immunotherapy.[5-8] For example, POLE and POLD1 mutations could serve as biomarkers for immunotherapy outcomes.[9,10] Alterations in the gene encoding ARID1A compromised MMR proteins and functioned as a biomarker for longer progression-free survival after anti-PD-1/PD-L1 immunotherapy.[11-14] Mutations in *TET1*, a DNA demethylase, were strongly associated with improved immunotherapy outcomes in patients receiving ICI treatment.[15]

Histone–lysine *N*-methyltransferase 2 (KMT2) family genes, also known as MLL genes, are frequently mutated in various types of cancer.[16] The KMT2 complexes methylate histone 3 lysine 4 (H3K4) to modulate DNA accessibility and transcription. In *Drosophila*, KMT2 proteins comprise three subgroups: Trx, Trr, and Set1. In mammals, there are two paralogs corresponding to each of the three subgroups: *Trx*-related *KMT2A* and *KMT2B*, *Trr*-related *KMT2C* and *KMT2D*, and *Set1*-related *KMT2F* and *KMT2G*.[17] A previous study showed that mutations in the KMT2 family (KMT2A, KMT2B, KMT2C, and KMT2D) were associated with favorable responses to immune checkpoint therapy in melanoma and non–small-cell lung cancer.[18] Rampias et al. demonstrated that KMT2C could regulate the expression of genes involved in DDR and DNA repair. Low KMT2C activity could lead to greater DNA damage and genomic instability.[19] In addition, loss of KMT2D might be associated with increased DNA damage and higher tumor mutation burden (TMB).[20] Another recent study showed that KMT2D-deficient mice tumors were characterized by significantly increased infiltration of T cells and antigen-presenting cells.[21] As KMT2C and KMT2D are paralogs to Trr and both play important roles in the DDR pathway, we hypothesized that *KMT2C* or *KMT2C/D* LOF variants might be a useful predictive biomarker for the efficacy of immunotherapy in different solid tumors. Here, for the first time, we systematically investigated the associations of *KMT2C/D* LOF variants with immunotherapy biomarkers (TMB, MSI-H, PD-L1 expression, and TILs) and clinical response to ICI therapy.

2 | MATERIALS AND METHODS

2.1 | Definition of *KMT2C/D* loss-of-function (LOF) variants

The *KMT2C/D* variants are referred to as genetic variants in the *KMT2C* or *KMT2D* genes. LOF variants were defined as nonsense, frameshift, splice site change within consensus regions, start lost, and stop lost/gained variants. Missense and inframe variants were excluded from the analysis.

2.2 | Study design and clinical cohorts

2.2.1 | The Cancer Genome Atlas (TCGA) cohort

TCGA cohort contained 4621 patients with eight cancer types (Table S2). Bladder cancer ($n = 411$), head and neck cancer ($n = 503$), non–small-cell lung cancer (NSCLC, $n = 1038$), colorectal cancer (CRC, $n = 507$), breast cancer ($n = 976$), melanoma ($n = 439$), hepatocellular carcinoma (HCC, $n = 359$), and glioma ($n = 388$) were included in the study. The whole-exome sequencing (WES) data and RNA sequencing (RNA-Seq) data were obtained using TCGA data portal. The mRNA expression values were calculated as fragments per kilobase of transcript per million mapped reads (FPKM). The relative proportions of 22 types of infiltrating immune cells were quantified using CIBERSORT (https://cibersort.stanford.edu/).[22] To confirm the TILs data in colorectal cancer, the tumor immune estimation resource (TIMER) algorithm database (https://cistrome.shinyapps.io/timer/) was also used to estimate immune cell populations.[23,24]

2.2.2 | MSKCC pan-cancer ICIs cohort

The MSKCC pan-cancer cohort contained 1524 patients who received ICIs therapy. Clinicopathologic and genomic data were

collected through the cBioPortal at https://www.cbioportal.org/study?id=tmb_mskcc_2018.[25] Samples of nine cancer types were selected: NSCLC (n = 344), melanoma (n = 313), bladder cancer (n = 211), renal cell carcinoma (n = 143), head and neck cancer (n = 129), esophagogastric cancer (n = 118), glioma (n = 116), CRC (n = 109), and breast cancer (n = 41) (Table S2). Some patients without genomic data or cancer-type information were excluded from the analysis. The OS data after ICI treatment (PD-1/PD-L1/CTLA-4/combination) were available in this cohort.

2.2.3 | Chinese cohorts

We enrolled, in total, 3427 Chinese patients (*KMT2C/D* LOF = 170; *KMT2C/D* non-LOF = 3257). Written consent was signed by all the patients. Bladder cancer (n = 31), gastric cancer (n = 269), NSCLC (n = 1080), CRC (n = 509), pancreatic cancer (n = 200), bile duct cancer (n = 122), melanoma (n = 130), HCC (n = 309), and glioma (n = 778) were enrolled (Table S2). The clinical and genetic characteristics of patients with *KMT2C/D* LOF variants are presented in Table S3. The formalin-fixed paraffin-embedded (FFPE) tissues from 14 CRC patients in this cohort were assessed by immunohistochemistry (IHC) staining using anti-CD3, anti-CD8, anti-CD68, and anti-FOXP3 antibodies.

To analyze the correlation between *KMT2C/D* LOF mutations and clinical response, 28 CRC patients treated with anti-PD-1-based combination therapies at the Harbin Medical University Cancer Hospital were involved. The flow chart of patient enrollment is shown in Figure S1. The tumor response was assessed by physicians using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 criteria. The overall response (ORR) rate was defined as the percentage of patients with the best overall response of complete or partial response (CR or PR).

2.2.4 | DNA sequencing

Targeted NGS (Onco PanScan panel) or WES were performed on matched tumor–blood specimens from patients in the Chinese cohort in a CAP-, CLIA-, and ISO15189-accredited laboratory (Genetron Health). The sequencing data were analyzed to characterize genomic alterations, microsatellite instability (MSI) status, and TMB. TMB was calculated as the number of non-silent somatic mutations (non-synonymous SNV; InDel, and splicing variants) per mega-base of genomic coding regions sequenced.

2.2.5 | Immunohistochemistry

Membranous expression of PD-L1 was assessed for patients in the Chinese cohort using an immunohistochemistry (IHC) assay (22C3 antibody).

FFPE tissues from 14 CRC patients were serially sectioned at 4-μm thickness. The primary antibodies (CD3: 1:400, Clone D7A6E, 85061s, Cell Signaling Technology; CD8: 1:100, Clone D8A8Y, 85336s, Cell Signaling Technology; CD68: 1:800, 76437s, Clone D4B9C, Cell Signaling Technology; FOXP3: 1:500, Clone 236A/E7, ab20034, Abcam) were incubated with the specimens. IHC assay was performed using the Leica BOND MAX System. The photographs were taken under an Olympus BX43 microscope and the cells on the whole slide level were quantified. Next, we assessed the positive rates of $CD3^+$, $CD8^+$, $CD68^+$, and $FOXP3^+$ cells, which were defined as the number of stained cells divided by the total number of immune cells, multiplied by 100%.

2.2.6 | Statistical analysis

For the Chinese cohort, the associations of *KMT2C/D* LOF variants with TMB, MSI-H, PD-L1 expression, and TILs levels were analyzed. The correlation between *KMT2C/D* LOF mutations and clinical response was analyzed in another cohort of 28 Chinese CRC patients. The relationships between *KMT2C/D* LOF variants and TMB, PD-L1 expression, TILs levels, and expression of immune-related genes were investigated in TCGA cohort. The MSKCC cohort was selected to analyze the association between *KMT2C/D* LOF variants and the efficacy of immunotherapy. All comparisons and statistical analyses were performed for all enrolled patients and each cancer type separately. The Kaplan–Meier method was used for survival analysis and the log-rank test method was used for assessing its statistical significance. Differences between groups were compared using the unpaired t-test or chi-squared test. A p-value of 0.05 was considered statistically significant.

3 | RESULTS

3.1 | The prevalence of *KMT2C/D* LOF variants among different tumor subtypes

The prevalence of *KMT2C/D* LOF variants across different cancer subtypes is summarized in Figure 1. In the Chinese cohort, the highest incidence rate of *KMT2C/D* LOF variants was found in bladder cancer (22.6%), followed by gastric cancer (7.8%) and colorectal cancer (6.7%). In the MSKCC cohort, *KMT2C/D* LOF variants were more abundant in bladder cancer (29.4%), colorectal cancer (21.1%), and head and neck cancer (14.0%). In TCGA cohort, *KMT2C/D* LOF variants were more common in the following cancer types: bladder cancer (24.1%), head and neck cancer (12.1%), and NSCLC (9.8%). Taken together, *KMT2C/D* LOF variants were more prevalent in bladder, head and neck, and colorectal cancer.

3.2 | *KMT2C/D* LOF variants were associated with increased TMB and level of MSI-H

Studies have shown that mutations in *KMT2C* and *KMT2D* play important roles in genomic instability and increased TMB.[19-21] To

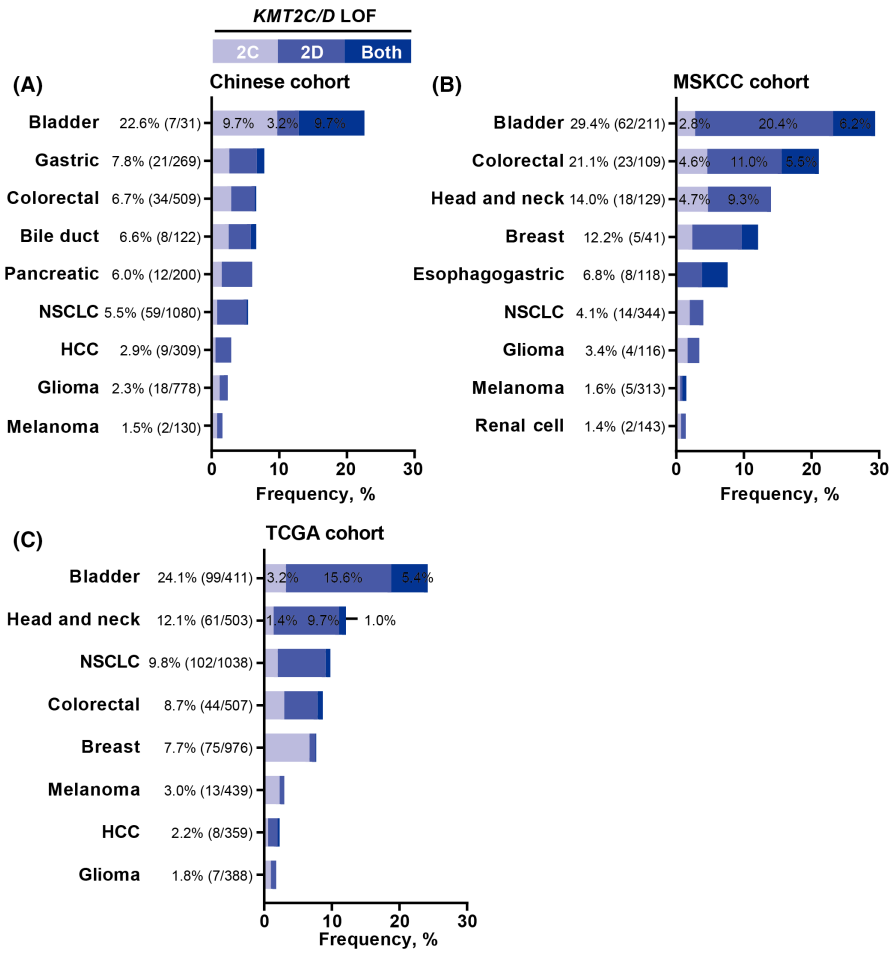

FIGURE 1 Prevalence of *KMT2C/D* LOF variants. Frequency of *KMT2C/D* LOF variants across different cancer types in the Chinese (A), MSKCC (B), and TCGA (C) cohorts.

investigate the relationships of *KMT2C/D* LOF variants with distinct predictive biomarkers for immunotherapy, we started with TMB, one of the most widely used immunotherapy biomarkers.[25] The median TMB was significantly higher in patients with *KMT2C/D* LOF variants compared with non-LOF variants in the Chinese cohort ($p < 0.0001$). The *KMT2C/D* LOF variants were also associated with higher TMB in all cancer types, except for bladder cancer, pancreatic cancer, HCC, and melanoma (Figure 2A, top panel). This finding was consistent with the conclusion in the MSKCC cohort, suggesting that the correlations between *KMT2C/D* LOF variants and higher TMB could be found for all enrolled patients ($p < 0.0001$) and all cancer types, with the exception of breast cancer and renal cell carcinoma (Figure 2A, middle panel). Furthermore, we also found associations between *KMT2C/D* LOF variants and higher TMB for all enrolled patients ($p < 0.0001$) and all types of cancer other than HCC in TCGA cohort (Figure 2A, bottom panel). The median TMBs for *KMT2C/D* LOF-mutated and non-LOF-mutated patients in three cohorts are presented in Table S2. Therefore, *KMT2C/D* LOF variants were associated with an increased TMB value in almost all types of cancer.

Next, the association between *KMT2C/D* LOF variants and MSI-H status was investigated in the Chinese cohort (Figure 2B). For all enrolled patients, 20.0% of patients were identified as MSI-H in the LOF group, whereas only 0.9% of patients had MSI-H tumors in the non-LOF group ($p < 0.0001$). Moreover, MSI-H was more prevalent in colorectal and gastric cancers. The proportions of MSI-H patients were significantly higher in the LOF groups in these two cancers ($p < 0.0001$, colorectal cancer; $p < 0.0001$, gastric cancer). To investigate whether the increased TMB in the *KMT2C/D* LOF group was due to the association between *KMT2C/D* LOF variants and MSI-H status, we evaluated the relationship between *KMT2C/D* LOF variants and TMB in patients with MSS tumors. The results showed that MSS patients with *KMT2C/D* LOF variants also had higher TMB compared with those without LOF variants ($p < 0.0001$) (Figure 2C). This suggested that an elevated TMB caused by *KMT2C/D* LOF appeared to be independent of MSI-H status.

3.3 | *KMT2C/D* LOF variants were associated with PD-L1 expression and levels of TILs in colorectal cancer

PD-L1 expression is one of the most widely studied biomarkers for response to ICIs. First, our study investigated whether *KMT2C/D* LOF variants were associated with PD-L1 expression in the Chinese cohort. The patients were divided into *KMT2C/D* LOF and non-LOF groups. The percentages of patients with PD-L1 TPS of more than 10% in these two groups were analyzed. The data indicated that the proportions of patients with PD-L1 TPS of more than 10% were

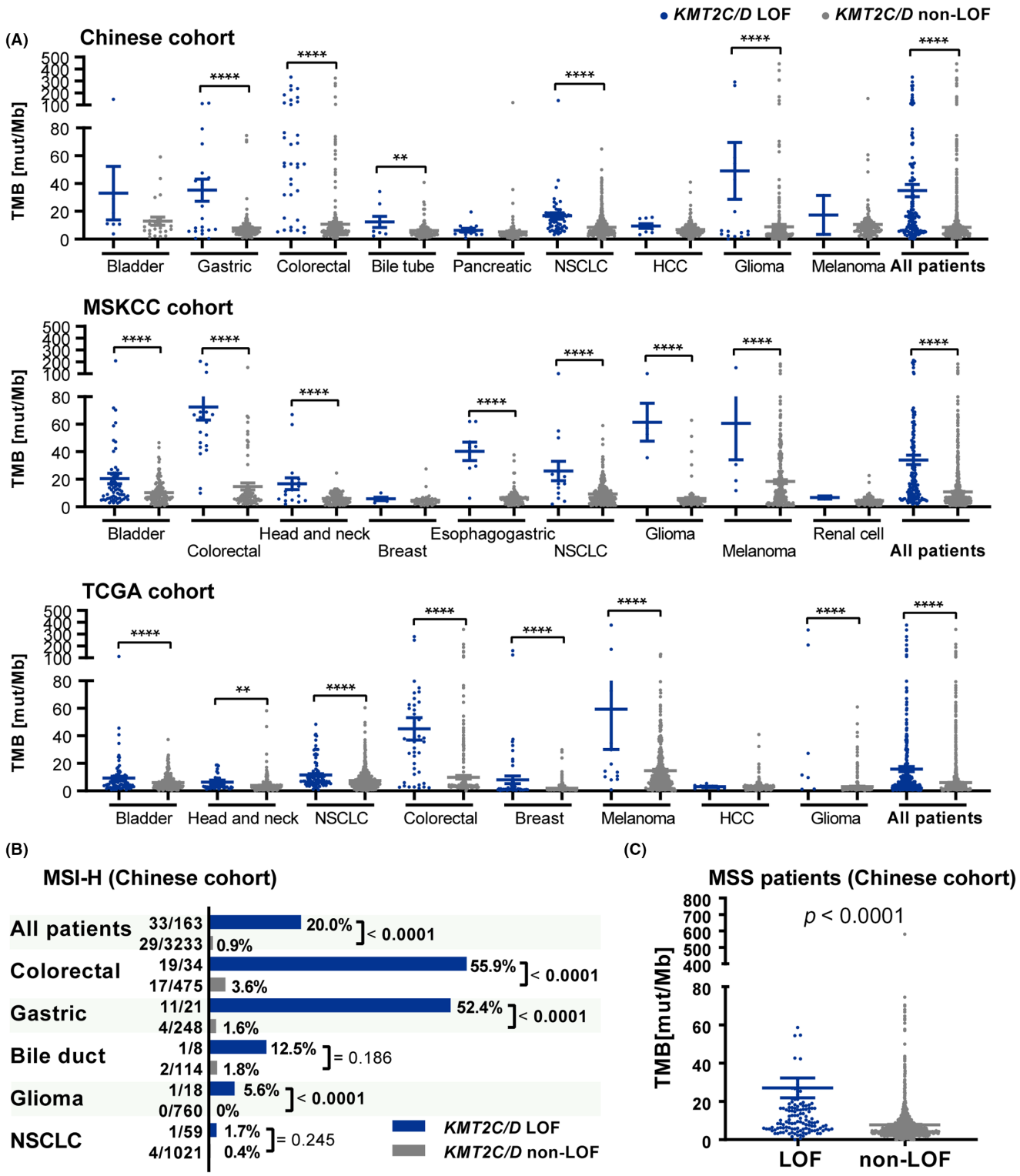

FIGURE 2 Association of *KMT2C/D* LOF with TMB or MSI-H status. (A) Comparisons of TMB between the *KMT2C/D* LOF and non-LOF groups across different cancers in the Chinese (upper panel), MSKCC (middle panel), and TCGA (bottom panel) cohorts. (B) Comparisons of the percentages of patients with MSI-H tumors between the *KMT2C/D* LOF and non-LOF groups in the Chinese cohort. (C) The associations of *KMT2C/D* LOF variants with TMB in patients with microsatellite stable (MSS) tumors in the Chinese cohort. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

higher in the LOF groups for all enrolled patients ($p = 0.024$), colorectal cancer ($p = 0.031$), and NSCLC ($p = 0.047$) (Figure 3A). Next, we analyzed the association between *KMT2C/D* LOF variants and PD-L1 expression in TCGA cohort. The result revealed that PD-L1 expression was significantly higher in the *KMT2C/D* LOF group compared with the non-LOF group for colorectal cancer ($p < 0.0001$). For

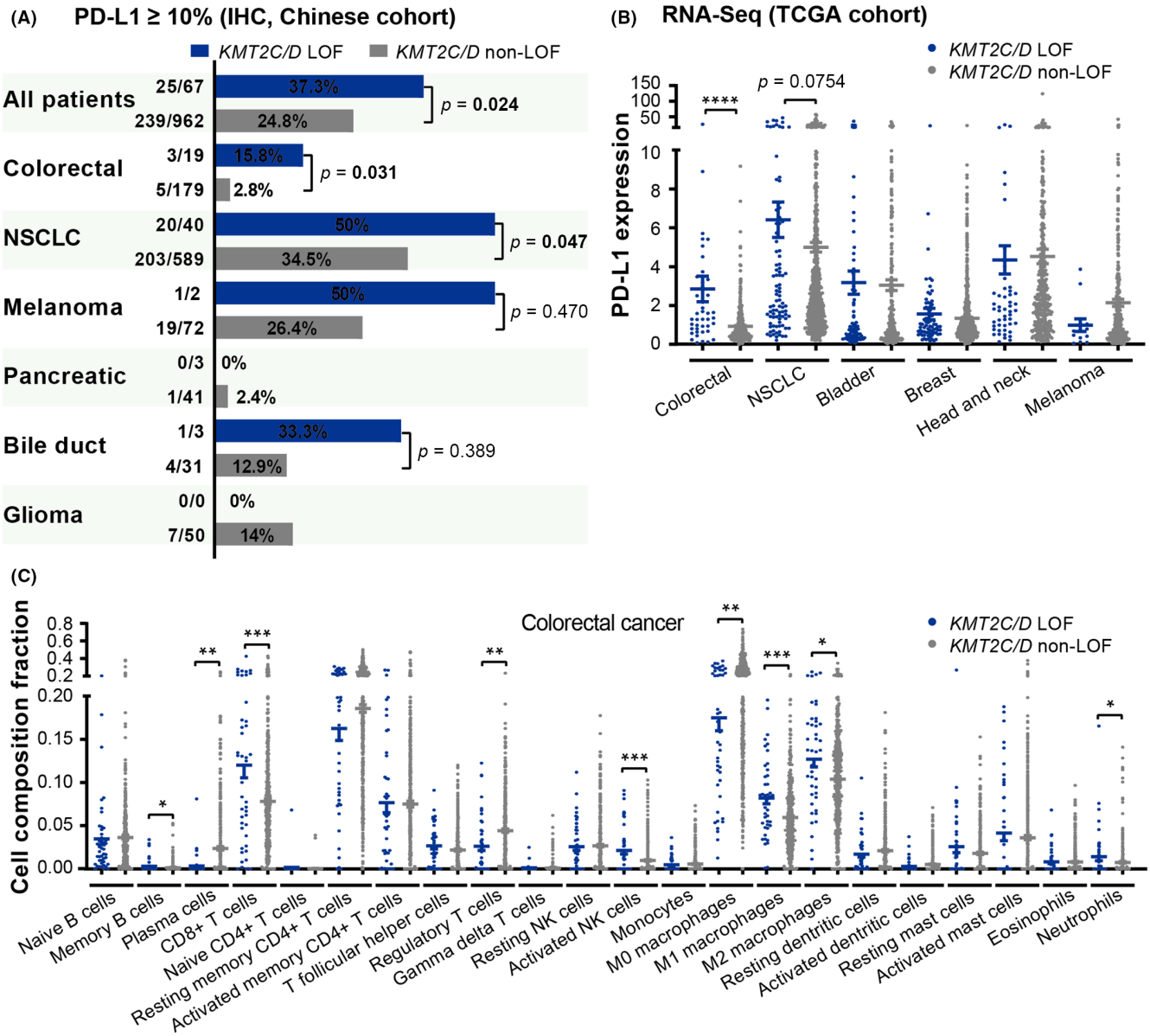

FIGURE 3 *KMT2C/D* LOF variants are associated with PD-L1 expression or immune cell infiltration in patients with colorectal cancer. (A) The percentages of patients with PD-L1 expression >10% in the *KMT2C/D* LOF and non-LOF groups were compared across different cancer types in the Chinese cohort using IHC. (B) Comparisons of PD-L1 expression between the *KMT2C/D* LOF and non-LOF groups in TCGA cohort with RNA-Seq. (C) Comparison of tumor-infiltrating leukocytes between the *KMT2C/D* LOF and non-LOF groups in colorectal cancer. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

patients with NSCLC, there was a trend suggesting that *KMT2C/D* LOF variants caused a significant increase in PD-L1 expression. However, this trend was not statistically significant ($p = 0.0754$) (Figure 3B). Taken together, these results showed that *KMT2C/D* LOF variants were associated with higher PD-L1 expression in colorectal cancer, rather than in other types of cancer.

To investigate whether there was an association between *KMT2C/D* LOF variants and the levels of TILs, The CIBERSORT tool was used to quantify the proportions of immune cells in TCGA cohort. Consistent with the results on PD-L1 expression, a significant association between *KMT2C/D* LOF variants and TILs was found in colorectal cancer. As shown in Figure 3C, analysis of TILs in colorectal cancer revealed that *KMT2C/D* LOF could increase infiltration in several types of immune cells, including $CD8^{+}$ T cells ($p < 0.001$), activated NK cells ($p < 0.001$), M1 macrophages ($p < 0.001$), M2 macrophages ($p < 0.05$), and neutrophils ($p < 0.05$). In addition, Tregs ($p < 0.01$) and M0 macrophages ($p < 0.01$) were dramatically decreased in the *KMT2C/D* LOF group. The relationship between *KMT2C/D* LOF variants and TILs was also analyzed in other cancer types (Figures S2 and S3). In NSCLC, tumors with *KMT2C/D* LOF variants were infiltrated with decreased Tregs ($p < 0.01$) and increased M1 macrophages ($p < 0.05$). However, the infiltration was less significant than in colorectal cancer. *KMT2C/D* LOF also resulted in increased infiltration of resting dendritic cells ($p < 0.05$) and

activated dendritic cells ($p < 0.0001$) in breast cancer. There were no significant differences in TILs infiltration between the *KMT2C/D* LOF and non-LOF groups for melanoma and glioma. In summary, the association of *KMT2C/D* LOF variants with TILs was mainly found in colorectal cancer. Next, IHC was performed with antibodies against CD3, CD8, CD68, and FOXP3 antigens on FFPE tissue sections of 14 patients in the Chinese cohort to validate the association of *KMT2C/D* LOF variants with TILs in colorectal cancer. The positive rates of $CD3^+$, $CD8^+$, $CD68^+$ and $FOXP3^+$ cells were calculated. As shown in Figure S4, the ratio of $CD3^+$ cells to immune cells was significantly higher in the *KMT2C/D* LOF group ($p < 0.05$). The ratio of $CD8^+$ cells to immune cells was also associated with *KMT2C/D* LOF although the *p*-value was slightly higher than 0.05 ($p = 0.0639$), which was consistent with the result produced by the CIBERSORT tool. CD68 has been widely used as a pan-macrophage marker. The macrophages were lower in the *KMT2C/D* LOF-mutated tissues. Due to the very low level of FOXP3 staining, no statistically significant difference in $FOXP3^+$ Treg cells was found between the LOF and non-LOF groups. In summary, the result indicated that *KMT2C/D* LOF variants might be associated with increased infiltration of $CD3^+$ T cells and $CD8^+$ T cells and decreased infiltration of total macrophages. The representative IHC images from four patients are shown in Figure S4.

3.4 | Association between *KMT2C/D* LOF variants and a better clinical benefit to ICIs in colorectal cancer

Using the MSKCC pan-cancer ICIs cohort, this study sought to evaluate whether *KMT2C/D* LOF variants could serve as a predictive biomarker for response to ICIs therapy (Figure 4A). For all enrolled patients, those who harbored *KMT2C/D* LOF variants had a relatively longer median OS compared with those without LOF variants, although the result was not statistically significant ($p = 0.0832$). Analysis of the predictive roles for each type of cancer revealed that *KMT2C/D* LOF had different predictive values in various cancer cell types. *KMT2C/D* LOF variants were associated with a prolonged OS in colorectal cancer ($p = 0.0485$), rather than in other cancer types. This suggested that *KMT2C/D* LOF variants might act as a predictive biomarker for ICIs therapy in colorectal cancer in this cohort. Moreover, the Kaplan–Meier curves for colorectal and breast cancers were also determined and the results are presented in Figure 4B,C. According to the results, there was an association between *KMT2C/D* LOF variants and a longer median OS in colorectal cancer. In contrast, *KMT2C/D* LOF variants did not correlate with prolonged OS in breast cancer. In summary, *KMT2C/D* LOF variants might serve as a predictive biomarker for ICIs in colorectal cancer.

In total, 28 CRC patients were also included for analyzing the predictive ability of *KMT2C/D* LOF mutations. Baseline characteristics are summarized in Table S1. Overall, seven patients achieved partial response (PR), 14 patients achieved stable disease (SD), and seven patients achieved progressive disease (PD), resulting in an ORR rate of 25% (7/28). Among the 28 patients, 4 patients had *KMT2C/D* LOF mutations. The ORR rate was significantly higher in the *KMT2C/D* LOF group than in the non-LOF group (100% versus 13%, $p = 0.002$). The tumors from four patients were MSI-H. Among 24 MSS patients, the *KMT2C/D* LOF mutation was also associated with a higher ORR rate (100% versus 5%, $p = 0.011$) (Figure 5). Together with the findings in the MSKCC cohort, these results indicated the robustness of *KMT2C/D* LOF mutations as a predictive biomarker for ICIs.

3.5 | Association of *KMT2C/D* LOF variants with the expression profile of immune-related genes and immune infiltration in colorectal cancer

In previous studies, 40 immune-related genes were collected and classified into three categories, including T-cell receptor, immune checkpoint, and T-effector and interferon-γ gene signature.[26,27] Therefore, further investigation was performed to determine the effect of *KMT2C/D* LOF on the expression levels of these genes in TCGA colorectal cancer cohort (Figure 6A–C). The results demonstrated that *KMT2C/D* LOF resulted in increased expression of all genes tested in colorectal cancer. Cytolytic activity score (CYT) is defined as the mRNA expression levels of granzyme A (*GZMA*) and perforin-1 (*PRF1*), which are upregulated upon $CD8^+$ T-cell activation.[26] The results suggested that *KMT2C/D* LOF could lead to an increased CYT score ($p < 0.0001$) (Figure 6D). To further confirm the TIL data in colorectal cancer, the TIMER web resource was also used to investigate the levels of infiltrating immune cells. The data showed that there were differences in four types of immune cells, including $CD8^+$ T cells ($p < 0.0001$), neutrophil cells ($p < 0.0001$), macrophages ($p < 0.05$), and dendritic cells ($p < 0.0001$) (Figure 6E). To conclude, *KMT2C/D* LOF could increase the expression of selected immune-related genes and the "CYT" score in colorectal cancer. Both CIBERSORT and TIMER results showed that *KMT2C/D* LOF resulted in a dramatic elevation of immunoactive TILs in colorectal cancer. It was indicated that *KMT2C/D* LOF might serve as a useful biomarker for immunotherapy response in colorectal cancer.

4 | DISCUSSION

In this study, it was identified that *KMT2C/D* LOF variants were abundant across many cancer types. In addition, this study established a strong association between *KMT2C/D* LOF variants and higher TMB in various types of cancer. These results are consistent with previous findings that indicated that KMT2C[19] and KMT2D[20] played important roles in the regulation of DDR gene expression. It was interesting to note that *KMT2C/D* LOF variants were associated with higher PD-L1 expression and TIL levels in colorectal cancer, rather than in other cancer types. More importantly, there was a statistically significant association between *KMT2C/D* LOF variants and the response to ICIs therapy only in colorectal cancer. Moreover, we found that *KMT2C/D* LOF variants were also associated with

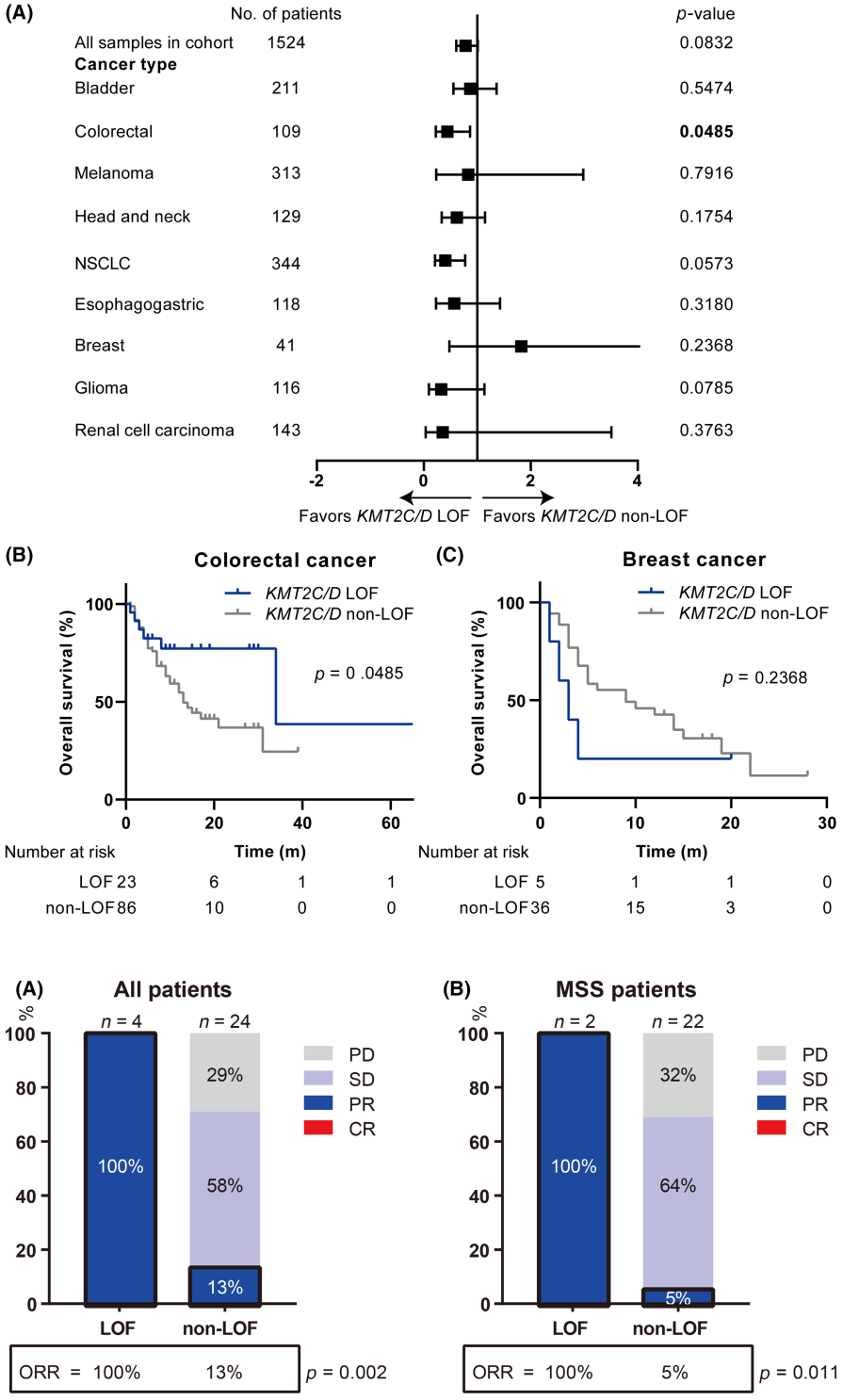

FIGURE 4 Patients with *KMT2C/D* LOF benefited from ICIs in colorectal cancer in the MSKCC cohort. (A) Forest plot for all patients in the whole cohort or different cancers. This study compared the OS between the *KMT2C/D* LOF and non-LOF groups. (B) Kaplan–Meier survival curves of OS comparing the *KMT2C/D* LOF and non-LOF groups in patients with colorectal cancer treated with ICIs. (C) Kaplan–Meier survival curves of OS in patients with breast cancer.

FIGURE 5 *KMT2D* LOF could predict the clinical response to ICIs. (A) Comparison of the clinical response in the LOF and non-LOF groups. (B) Comparison of the clinical response for MSS patients in the LOF and non-LOF groups.

higher ORR rates in an advanced colorectal cancer cohort with ICI treatment. For MSS patients, it also has a positive predictive value. According to the data, *KMT2C/D* LOF variants had a predictive role in the efficacy of ICIs treatment in colorectal cancer. In addition, the predictive role of *KMT2C/D* LOF variants on ICI efficacy was in keeping with their association with TIL levels.

Analysis of TILs in colorectal cancer by CIBERSORT showed that *KMT2C/D* LOF variants were positively related to $CD8^+$ T cells, activated NK cells, M1 macrophages, M2 macrophages, and neutrophils and negatively correlated with M0 macrophages and Tregs. The IHC staining data indicated increased infiltration of $CD3^+$ T cells and $CD8^+$ T cells and decreased infiltration of total macrophages in *KMT2C/D* LOF-mutated tissues. Although the difference was found for macrophages, the results suggested that *KMT2C/D* LOF variants were associated with high levels of $CD8^+$ T cells and immunoactive tumor microenvironment. In NSCLC, *KMT2C/D* LOF

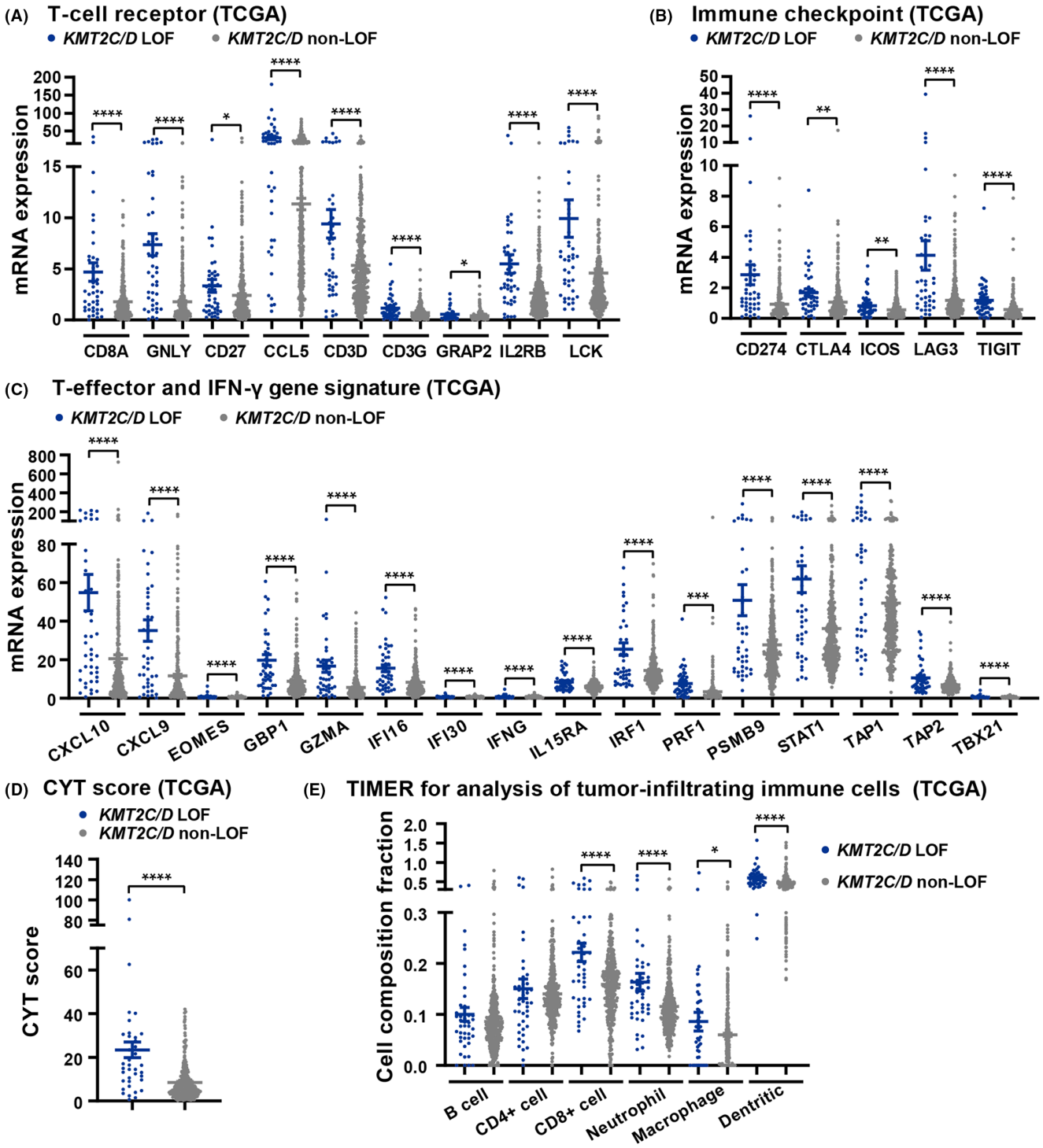

FIGURE 6 Comparisons of immune-related genes expression between the *KMT2C/D* LOF and non-LOF groups in colorectal cancer. These genes were classified into three classes, including T-cell receptor (A), immune checkpoint (B), T-effector and IFN-γ gene signature (C). (D) Correlations between CYT score and *KMT2C/D* LOF variants. (E) TIMER analysis was used to compare the abundance of six immune cell types, including B cells, $CD4^+$ T cells, $CD8^+$ T cells, neutrophils, macrophages, and dendritic cells. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

variants were found to be associated with decreased infiltration of Tregs and increased infiltration of M1 macrophages. However, the link between *KMT2C/D* LOF variants and TILs levels was not very strong. Consistently, the NSCLC patients who harbored *KMT2C/D* LOF variants also had a relatively longer OS compared with those without LOF variants. However, the difference was not statistically significant ($p = 0.0573$) (Figure 4A). In breast cancer, *KMT2C/D* LOF variants did not affect the infiltration of immune cells. As well, the

opposite trend showed that patients with *KMT2C/D* LOF variants had a shorter OS ($p = 0.2368$) (Figure 4A,C). To sum up, the predictive value of *KMT2C/D* LOF variants on the efficacy of ICIs was strongly dependent on the affected infiltration of immune cells.

Consistent with a previous study demonstrating that *KMT2D* mutations were associated with increased infiltration of T cells and antigen-presenting cells,[21] our study indicated that *KMT2C/D* LOF mutations were correlated with increased infiltration of immune cells in a cancer type-dependent manner. Thorsson et al. showed that both the level and composition of the tumor immune infiltrate varied substantially across cancer types. In addition, the impact of tumor immune infiltrates on clinical outcomes for patients also differed between cancer types.[28] A review study presented that, not only the tumor genetics but also the tissue-specific features, such as blood, lymphatic vasculature, and fibroblast, could shape tumor tissue immune cell infiltrates.[29] Because the mechanisms regulating the infiltration of immune cells are very complicated and the tumor-infiltrating immune cells differ across cancer types, the association of *KMT2C/D* LOF variants with the levels of TILs and the clinical outcomes of ICIs varied significantly across cancer types. Similar results were also presented for the *ARID1A* mutations, another potential predictive marker of response to ICIs.[30,31] Different variables have been previously reported to be associated with anti-PD-1/PD-L1 response, such as TMB, the expression of PD-L1, and $CD8^+$ T-cell abundance. But it is difficult to accurately predict the response to immunotherapy in all types of cancer by relying on one biomarker alone. A better approach is to evaluate the performance of each class of variables and their combinations in predicting the efficacy of ICIs.[32]

The previous study identified that KMT2D was a major regulator of ICIs response. *KMT2D* loss sensitized tumors to immune checkpoint blockade in mice bearing sgKmt2d transducing cancer cells, including bladder cancer, breast cancer, melanoma, and lung cancer cells. In two small cohorts of bladder cancer patients, they found that *KMT2D* mutant bladder cancer patients were more likely to respond to anti-PD-L1 therapy.[21] In the present study, the results of the MSKCC cohort did not show any relationship between *KMT2C/D* LOF variants and the efficacy of ICIs in bladder cancer. Therefore, another cohort is needed to assess the predictive role of *KMT2C/D* LOF variants in bladder cancer. Another recent study reported that *KMT2A/C* mutations functioned as a potential predictive biomarker for immunotherapy.[33] While we addressed the predictive roles of LOF variants in *KMT2C* and *KMT2D* genes, which are orthologous to *Drosophila* Trr. Beyond that, we also investigated the relationships between *KMT2C/D* LOF variants and TMB, MSI-H, PD-L1, and TILs. Most of all, we found that the prediction value of *KMT2C/D* LOF variants relied on their effect on the infiltrating immune cells.

In summary, the present study identified a novel and potential predictive biomarker for ICI efficacy in colorectal cancer. Furthermore, it was found that the prediction value of *KMT2C/D* LOF variants was dependent on their effect on the infiltrating immune cells. In addition, the result indicated that any predictive biomarker could not be used in all cancer types.

ACKNOWLEDGMENTS

The authors are grateful to all the investigators involved and the patients who gave their consent to present data.

FUNDING INFORMATION

National Natural Science Foundation of China (Nos. 81872427, 82173233, 82102988); Applied Technology Research and Development Program of Heilongjiang Province (No. GA19C002).

CONFLICTS OF INTEREST

The authors have no conflict of interest.

ETHICS STATEMENT

This study was in accordance with the ethical standards of the institutional ethics committee.

Informed consent: Informed consent was obtained from all individual participants in this study.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Liu R, Niu Y, Liu C, et al. Association of *KMT2C/D* loss-of-function variants with response to immune checkpoint blockades in colorectal cancer. *Cancer Sci*. 2023;114:1229-1239. doi:10.1111/cas.15716