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Brief Communication

RNF43 mutation as a predictor of immunotherapeutic efficacy in colorectal cancer

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Abstract: *RNF43* is a tumor suppressor for various cancers and is considered to drive carcinogenesis when mutated. However, the correlation between *RNF43* mutation and colorectal cancer (CRC) immunotherapy remains unreported. We evaluated the role of *RNF43* using publicly available data from the Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering Cancer Center (MSKCC). In addition, further analysis was performed on an internal validation cohort (hcohort). The mutant profiles of *RNF43* were analyzed in 873 Chinese CRC patients. The relationship between clinical pathologic features and *RNF43* were analyzed using the two-sided chi-squared test or the Fisher exact test. Clinicopathologic characteristics were associated with overall survival using Cox regression and the Kaplan-Meier method. We found that *RNF43* mutation was significantly associated with high TMB and high MSI score (all *p*-values < 0.05) in the MSKCC cohort. Additionally, *RNF43* mutation was found to be enriched in MSI instability. Kaplan-Meier survival analysis revealed that patients with *RNF43* mutation had better OS compared to *RNF43* wild-type (not reached vs. 13 months, HR, 0.12; 95% CI 0.03 to 0.49; P = 0.0034). However, no association was observed between *RNF43* and OS in the TCGA cohort (HR, 1.83; 95% CI 0.66 to 5.07; P = 0.2479). Our CRC hcohort confirmed the significance of *RNF43* mutation in predicting better clinical outcomes, including ORR (45% vs. 21%, P = 0.0468). *RNF43* mutation correlated with a high tumor mutation burden (P < 0.001). The mutation frequency of *RNF43* in CRC patients was 8.4% (73/873); *RNF43 G659Vfs*41* was found to be the most frequent mutation site. In patients with *RNF43* mutations, *TP53*, *KRAS,* and *TGFBR2* were genes with a high frequency of mutations. Compared with *RNF43* wild-type patients, those with *RNF43* mutations had a higher TMB score and a greater proportion of MSI-H, but no difference in PD-L1 expression. Moreover, the content of immune-related B cells, CD8+ T cells, neutrophils, and dendritic cells was higher in the *RNF43* mutant group than in the wild-type group. Our results suggest that *RNF43* mutation may correlate with better OS in CRC patients receiving PD-1/PD-L1 inhibitors. The exact mechanisms underlying *RNF43* require further investigation.

Keywords: *RNF43*, colorectal cancer, immunotherapy, tumor mutation burden, CD8+ T cell

Introduction

Colorectal cancer (CRC) is one of the most common cancers worldwide, ranking in the top three for both incidence and mortality rates [1, 2]. Although increased colonoscopy screening and removal of precursor lesions have improved the incidence and prognosis of CRC, a significant proportion of patients exist with advanced disease, and the 5-year survival rate for metastatic CRC remains below than 15% [3]. The development of immunotherapy, however, has shown promising success in enhancing long-term survival in previously refractory solid tumors, including CRC [4]. Currently, several immune checkpoint inhibitors, such as pembrolizumab and combined nivolumab and ipilimumab therapy, have demonstrated prom-

ising efficacy and have been approved by the United States Food and Drug Administration (FDA) for treating advanced CRC tumors with high levels of microsatellite instability (MSI-H) or mismatch repair deficiency (dMMR) [5, 6]. While MSI/MMR status is the only well-recognized biomarker for immunotherapy in CRC, only a small proportion of CRC patients are MSI-H/dMMR, and some MSI-H/dMMR patients may still exhibit primary or acquired resistance to immunotherapy [6, 7]. The independent predictive efficacies of other potential biomarkers, such as tumor mutation burden (TMB) and PD-L1 expression, remain controversial in CRC [8, 9]. There is a pressing need for more effective biomarkers to guide the immunotherapy treatment of CRC patients.

RNF43 (Ring Finger Protein 43) encodes an E3 ubiquitin-protein ligase that functions to downregulate the Wnt signaling pathway by inducing degradation of the Wnt receptor Frizzled through ubiquitination [10]. *RNF43* mutations have been found to be frequently mutated in various cancer types, including CRC, gastric cancer, pancreatic cancer, and ovarian cancer [11-13]. Loss-of-function *RNF43* mutations result in enhanced Wnt signaling and may represent a distinct molecular subtype with aggressive tumor biology [14]. In CRC, two truncating mutations of *RNF43*, *p.G659fs* and *p.R117fs*, have been frequently observed, with the former accounting for nearly 8% of all patients and being especially enriched in MSI-H tumors [15]. Furthermore, *RNF43* mutation combined with *BRAF V600E* mutation has been observed to promote tumor development and is significantly associated with worse survival in right-sided CRC [16]. However, the clinical significance of *RNF43* mutations in CRC and their association with CRC immunology remain largely unexplored.

In this study, we investigate the role of *RNF43* mutations in CRC survival based on two public datasets, the Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering Cancer Center (MSKCC), combined with an in-house Chinese CRC dataset containing 60 patients. The mutation characteristics of *RNF43* in CRC were characterized, and its predictive value for CRC immunotherapy efficacy was explored. In addition, we used a lager sample cohort (3Dcohort) to explore the mutation frequency and profile of *RNF43* in Chinese CRC patients. Moreover, several immune-related characteristics, including MSI status and TMB, were further analyzed to determine their correlation with *RNF43* mutations in CRC. We aim to provide a comprehensive overview of *RNF43* mutations and propose a potential predictor for immunotherapeutic efficacy in CRC.

Materials and methods

Clinical cohorts and public cohorts

Genomic and clinical data of CRC patients administrated with anti-PD-L1 or anti-CTLA-4 antibodies were retrieved from publicly MSKCC accessible data. The genomic, survival, and mRNA data of 457 patients with CRC were obtained from TCGA (www.cbioportal.org). Patients with advanced CRC treated with PD-1/PD-L1 inhibitors between February 2016 and January 2020 were included in our in-house cohort. All patients underwent genomic profiling (150-gene panel) of tumor tissue prior to immunotherapy. Of these, 26 tissue samples failed quality control and were replaced with blood samples for testing. The following clinical characteristics before therapy were recorded: age, sex, histological type, tumor location, response to therapy, and survival. All human samples were collected and used in accordance with the Declaration of Helsinki Principles and approved by Shenzhen People's Hospital (LL-KY-2021753). All participating patients provided written consent.

As for the 3Dcohort, 873 CRC patients were included from January 2017 to January 2020. Tumor tissue samples (self-blood negative control) of pan-cancer patients were analyzed by NGS for detection the *RNF43* mutations using a well-designed 150-gene panel on the Illumina HiSeq sequencer (Illumina, San Diego, CA) with 800× sequencing depth in a College of American Pathologists (CAP) and Clinical Laboratory Improvement Amendments (CLIA) certified laboratory (3D Medicine Inc., Shanghai, China). Somatic alterations were identified, and clinical information, including age, gender, and tumor histology, was collected. Additionally, TMB was defined as the number of nonsynonymous somatic SNVs and indels in examined coding regions, excluding driver mutations. All

SNVs and indels in the coding region of targeted genes, including missense, silent, stop gain, stop loss, in-frame, and frameshift mutations, were considered.

DNA extraction, library preparation and DNA sequencing

Experimental methods for next-generation sequencing (NGS), such as DNA extraction, library preparation, and DNA sequencing, are described in our previous work [17]. FFPE tissue sections were evaluated for tumor cell content using hematoxylin and eosin (H&E) staining. Only samples with a tumor content of ≥ 20% were eligible for subsequent analyses. Blood was centrifuged in a Streck tube at 1600 g for 20 min at room temperature to separate plasma. Then, gDNA was extracted from tumor FFPE tissue and white blood cells, respectively, following a standard protocol using the DNeasy tissue or blood kit (Qiagen). Libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems) as per the manufacturer's protocol. The concentration and size distribution of each library were determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific) and a LabChip GX Touch HT Analyzer (PerkinElmer), respectively. Single nucleotide variations (SNV), insertions/deletions, copy number variations (CNV), and gene fusions were assessed. Germline alterations were excluded.

Assessment of immune signatures

Based on gene mutation analysis, the infiltration level of immune cells in CRC was analyzed using the TIMER database (https://cistrome.shinyapps.io/timer/) [18]. The gene name *RNF43* under the mutation module was used to determine the relationship between *RNF43* mutation and the level of immune cell infiltration. The "CIBERSORT" (R package) was used to determine the proportion of 22 tumor-infiltrating immune cells (TIICs) in each sample [19]. The proportion of 22 TIICs in each tissue was predicted based on its GEPs. Normalized CRC GEPs were transformed into the proportion of 22 TIICs. The relative expression of 22 TIICs in each sample was determined.

Statistical analyses

Overall survival (OS) was calculated from the date of first line therapy administration to the date of death from any cause. Survival was illustrated using Kaplan-Meier curves, with *P*-values determined by a log-rank test. Hazard ratio (HR) was determined through the univariate and multivariate Cox regression. Variables of interest with significant *P*-values were included in multivariate logistic regression. Continuous variables were compared using the Mann-Whitney U test. For all analyses, *P*-value < 0.05 was considered statistically significant, with a confidence interval of 95% (95% CI). All statistical analyses were performed using SPSS22.0 software (SPSS Inc., Chicago, IL, USA).

Results

Association of RNF43 mutation with improved OS in CRC patients undergoing immunotherapy

Detailed baseline characteristics of CRC patients in two independent cohorts (MSKCC and TCGA) are summarized in **Table 1**. In the MSKCC cohort, 21 patients (19%) harbored an *RNF43* mutation (RNF43-MUT), while 88 were *RNF43* wild-type (RNF43-WT). The TCGA cohort had 45 patients (9%) with RNF43-MUT.

We first analyzed the association in the MSKCC cohort to explore the correlation between RNF43-MUT and the efficacy of immune checkpoint blockades (ICBs) in CRC patients. As shown in **Figure 1A**, *RNF43* mutation was significantly associated with better OS. The median OS for RNF43-MUT vs. RNF43-WT patients were not reached and 13.0 months, respectively (HR = 0.12; 95% CI, 0.03-0.49; *P* = 0.0034). RNF43-MUT patients also exhibited significantly higher MSI scores than RNF43-WT patients (**Figure 1B**, *P* < 0.0001). Notably, all patients with MSS type were found in the RNF43-WT group (**Figure 1C**). However, in the TCGA cohort of CRC patients not treated with ICBs, *RNF43* mutation was not associated with a better OS benefit. The median OS for RNF43-MUT vs. RNF43-WT patients were 70.1 months vs. 92.7 months (HR = 1.32; 95% CI, 0.71-2.46; *P* = 0.3805, **Figure 1D**).

Univariate and multivariable regression analyses of OS in the MSKCC cohort are detailed in **Table 2**. Confounders, including age, sex, drug type, tumor location, TMB, MSI, and *RNF43* mutations, were analyzed. Univariable analysis

Table 1. Baseline characteristics in MSKCC and TCGA cohorts

Characteristics	MSKCC cohort	TCGA cohort
Age, n (%)		
≤ 60	71 (65%)	174 (35%)
> 60	38 (35%)	329 (65%)
Sex, n (%)		
Female	48 (44%)	237 (47%)
Male	61 (56%)	266 (53%)
Cancer type, n (%)		
Colon Adenocarcinoma	87 (80%)	318 (63%)
Rectal Adenocarcinoma	16 (15%)	131 (26%)
Mucinous Adenocarcinoma of the Colon and Rectum	4 (4%)	53 (11%)
Signet Ring Cell Adenocarcinoma of the Colon and Rectum	4 (4%)	0
Colorectal Adenocarcinoma	1 (1%)	0
Drug type, n (%)		
Combo	10 (9.1%)	/
CTLA4	1 (0.9%)	/
PD-1/PDL-1	98 (90%)	/
Gene, n (%)		
RNF43 mutation	21 (19%)	45 (9%)
RNF43 wild-type	88 (81%)	458 (91%)
Sample type, n (%)		
Metastasis	59 (54%)	0
Primary	50 (46%)	503 (100%)

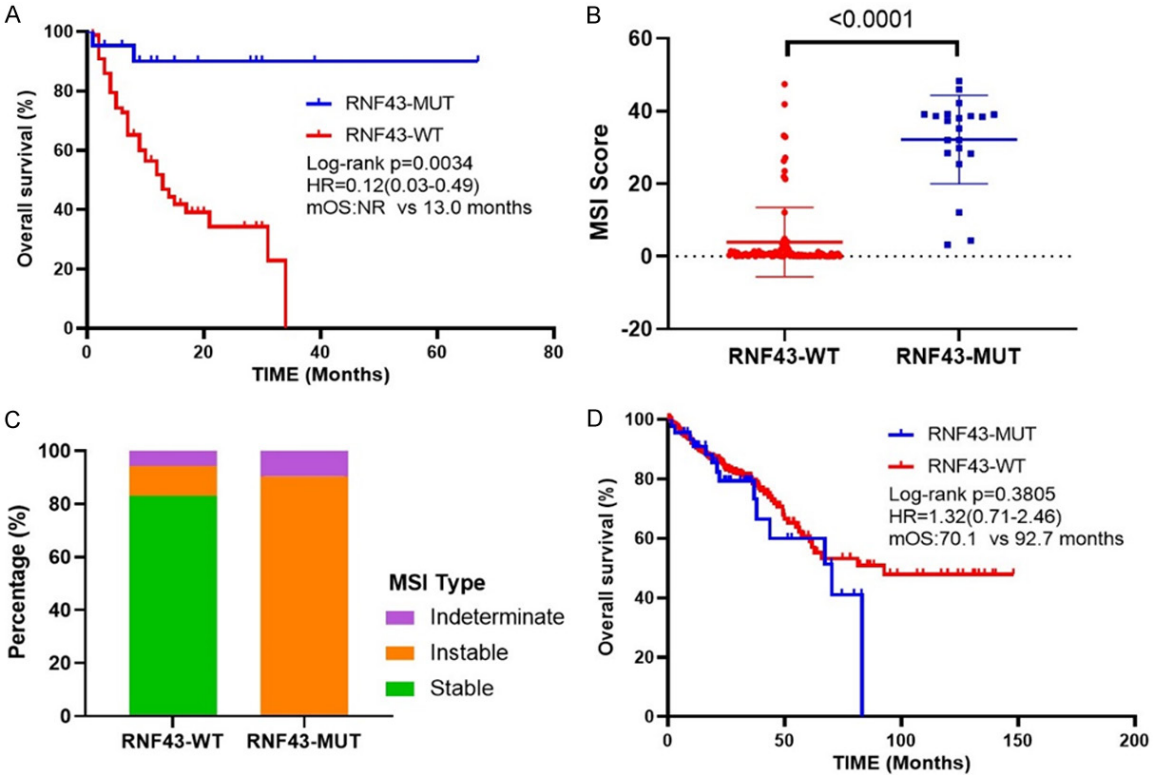

Figure 1. Association between *RNF43* mutation and OS in CRC patients. (A) Kaplan-Meier survival curves of OS comparing CRC patients with RNF43-MUT and wild-type in MSKCC cohort. Comparison of MSI score (B) and MSI type (C) between RNF43-MUT and RNF43-WT CRC patients in MSKCC. (D) Kaplan-Meier survival curves of OS comparing CRC patients with RNF43-MUT and wild-type in TCGA cohort. OS: overall survival. MSI: microsatellite instability.

Table 2. Univariate and multivariate analyses of overall survival in MSKCC cohort

Parameter	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Sex						
Male vs. Female	0.517	0.283-0.942	0.031	0.441	0.239-0.817	0.009
Age						
> 60 vs. ≤ 60	0.506	0.255-1.003	0.051			
RNF43 gene						
MUT vs. WT	0.122	0.032-0.552	0.006	0.128	0.025-0.747	0.022
Tumor location						
Rectum vs. others	0.858	0.337-2.185	0.748			
Drug type						
PD-L1/1 vs. others	0.992	0.305-3.224	0.989			
TMB status						
> median vs. ≤ median	0.628	0.340-1.158	0.136			
MSI status						
MSI-H vs. MSS	0.264	0.103-0.678	0.006	0.716	0.244-2.104	0.544

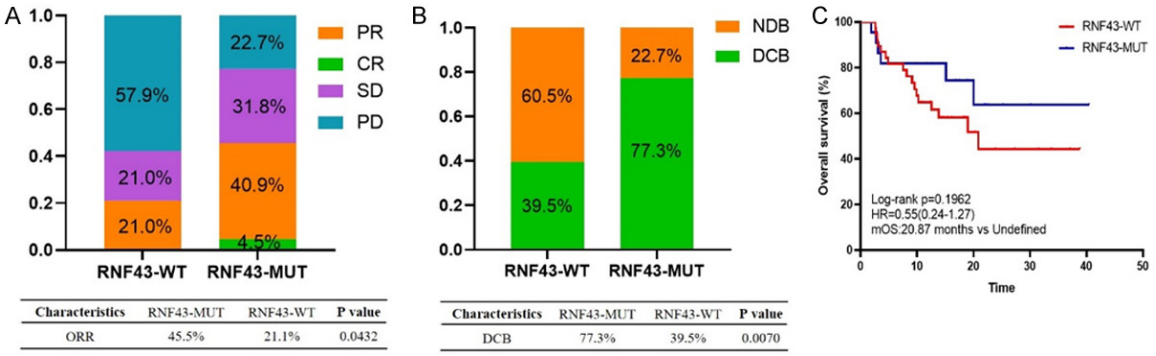

Figure 2. Association between *RNF43* mutation and OS in in-house cohort. Comparison of ORR (A) and DCB (B) between RNF43-MUT and RNF43-WT CRC patients. (C) Kaplan-Meier survival curves of OS comparing CRC patients with RNF43-MUT and wild-type. OS, overall survival; CR, Complete response; DCB, Durable clinical benefit; NDB, No durable benefit; PD, Progressive disease; PR, Partial response; SD, Stable disease.

revealed significant association between better OS and male, MSI-H, and *RNF43* mutation. The multivariable analysis identified *RNF43* mutation as an independent predictor for OS (HR = 0.13; 95% CI, 0.025-0.747; *P* = 0.022). Collectively, these findings suggest that RNF43-MUT could be a potential predictor for ICBs efficacy in CRC.

Association between RNF43 mutation and better OS benefit in our in-house cohort who received immunotherapy

The basic characteristics of our in-house ICBs treatment cohort (N = 60) are detailed in Table S1. Of these, 22 patients had RNF43-MUT, comprising 20 cases of colorectal cancer, one case of rectal cancer, and one case of ileocecal cancer, accounting for 36.7% of the total cohort. In this CRC cohort, 36 of the 60 patients (60.0%) were male, with a median age of 47 years (range: 15-77 years). No significant clinical differences were observed between the RNF43-MUT and RNF43-WT groups. Based on RECIST 1.1, the overall response was evaluable. Notably, the ORR for patients with RNF43-MUT was more than double that of RNF43-WT patients (45.5% vs. 21.1%, *P* = 0.0468, **Figure 2A**). For DCB, 77.3% of RNF43-MUT patients underwent ICBs treatment, compared to 39.5% of RNF43-WT patients (*P* = 0.0070, **Figure 2B**). While survival of RNF43-MUT CRC patients was superior to that of RNF43-WT patients, with a

median OS of undefined vs. 20.87 months, this was not statistically significant (P = 0.1962, **Figure 2C**). These findings demonstrate the predictive value of *RNF43* mutation for ICBs treatment.

We analyzed differential expression (DE) between RNF43 mutations and the RNF43 wild-type group in the hcohort. *RNF43* mutation was more likely to co-occur with mutations in *TGFBR2*, *ACVR2A*, *RAD50*, *BRCA1*, and *RB1* (Figure S1A). Moreover, the DEs were most significantly enriched in human papillomavirus infection, cancer pathways, and the PI3K-Akt signaling pathway (Figure S1B). Examining DEs, biological process (BP) terms are most significantly enriched in cellular metabolic process, metabolic process, and macromolecule biosynthetic process. Cellular component (CC) terms are most significantly enriched in intracellular, membrane-bounded organelle, and intracellular organelle. For molecular function (MF), binding, protein binding, and organic cyclic compound binding are mostly enriched (Figure S1C).

KDR-MUT was indicative of an immune-hot status

To further understand the underlying mechanism of the association between *RNF43* mutation and better clinical outcomes in CRC patients who received ICBs therapy, the impact of *RNF43* mutation on TMB or immune signatures was explored. **Figure 3A-C** demonstrate that *RNF43* mutation was associated with higher TMB both in the MSKCC cohort and our in-house CRC cohort ($P < 0.0001$). We subsequently analyzed data from 22 TIICs, comparing mRNA expression of immune related genes between *RNF43*-MUT and RNF43-WT patients. By using CIBERSORT, we found that activated NK cells and CD8+ T cells were more abundant in RNF43-MUT tumors. Furthermore, B cell, neutrophil cell, and dendritic cell infiltration increased (**Figure 3D**).

Moreover, PD-L1 (CD274) and CTLA4 were significantly up-regulated in RNF43-MUT tumors (**Figure 3E**). Additionally, RNF43-MUT tumors had a higher expression of MHC I- and II-associated antigen-presenting molecules than RNF43-WT tumors (**Figure 3F**, **3G**). These results suggest that RNF43-MUT is associated with a hot immune status and enhances the efficacy of ICBs.

Mutational profiles of RNF43 in 3Dcohort

To investigate the mutation profile of *RNF43* in Chinese CRC population, 873 Chinese CRC patients with 150 cancer-related genes detected by NGS were included in this study, including 513 patients with CRC and 315 patients with rectal cancer. A total of 73 (8.4%) patients with RNF43-MUT were identified (Table S2); this was significantly lower than both the MSKCC cohort and the internal cohort (MSKCC cohort: 8.4% vs. 19.3%, $P = 0.0008$; in-house cohort: 8.4% vs. 36.7%, $P < 0.0001$), and similar to those of the TCGA cohort (8.4% vs. 9.0%, P = 0.7644). These results might be attributed to the specific cohort under study.

The genomic mutational landscape of 73 patients with *RNF43* mutation is presented in **Figure 4A**. *TP53* was the most frequently mutated gene, followed by *KRAS*, *TGFBR2* and *MSH3*. In addition, 21 (22.3%) patients were confirmed harboring a *p.G659VFS*41* mutation (**Figure 4B**). The proportion of other major *RNF43* mutations were *p.R132** (7.4%), *p.R145** (6.4%), and *p.R117Afs*41* (2.1%, Table S3). *RNF43* mutations were associated with higher MSI-H in CRC patients (41.1% vs. 3.1%, $P < 0.0001$, **Figure 4C**), but not with PD-L1 status ($P = 0.166$, **Figure 4D**).

Discussion

In the present study, *RNF43* mutations were identified for first time as a positive factor for enhanced clinical benefit in CRC patients undergoing ICB treatment. Patients who did not undergo ICB treatment did not observe a clinical benefit for OS. Both univariate and multivariate analyses further established RNF43 mutations as independent positive predictors in the MSKCC cohort. Exploratory analyses suggest that a higher TMB and elevated expression of genes related to MHC I and II-associated antigen-presenting molecules might be a potential mechanism of the predictive value of *RNF43* mutations in CRC populations. GSEA also revealed that RNF43-MUT tumors have abundant immune features and microenvironment cell populations, further demonstrating that RNF43-MUT was associated with the hot tumor microenvironment. These results sug-

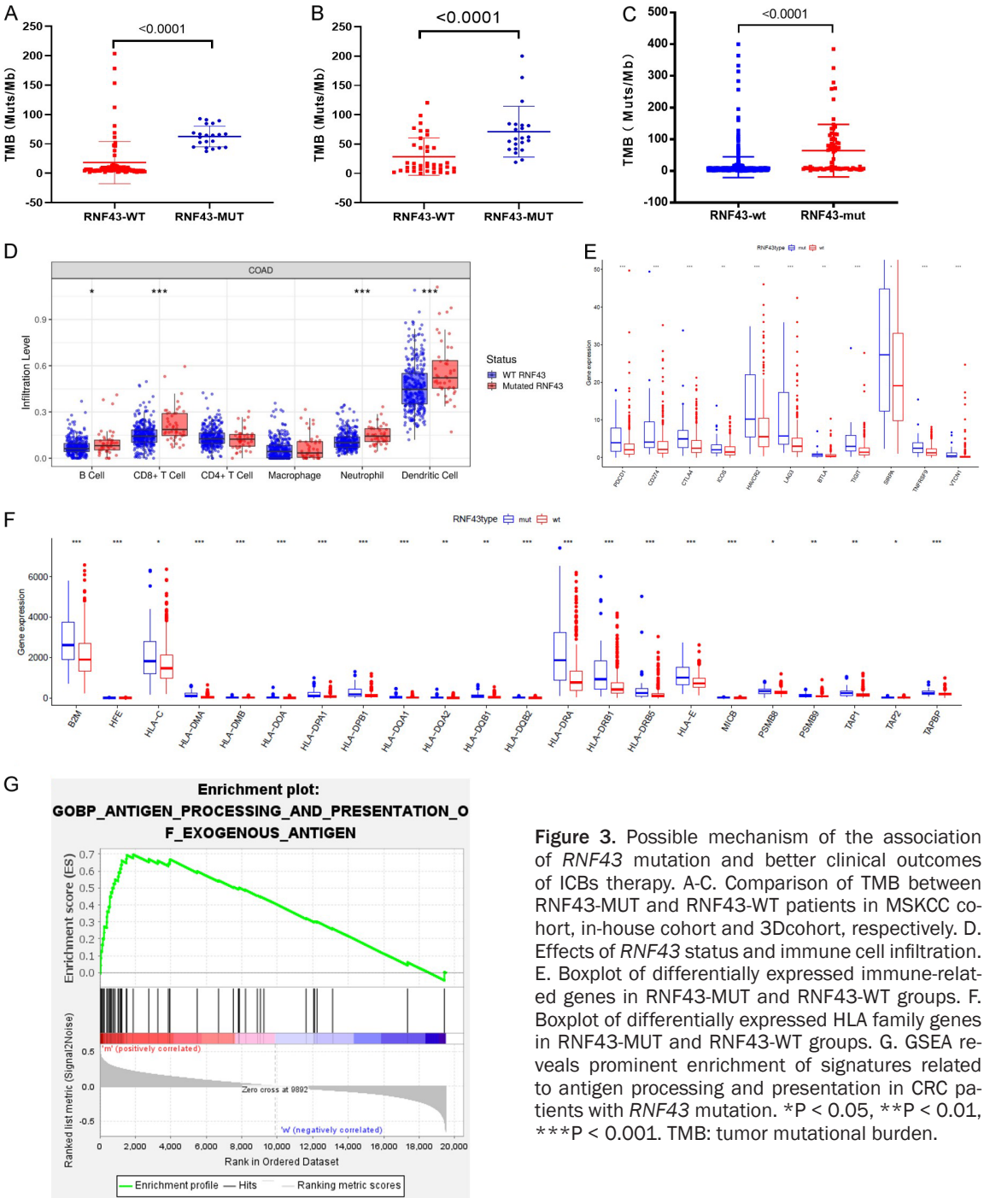

Figure 3. Possible mechanism of the association of *RNF43* mutation and better clinical outcomes of ICBs therapy. A-C. Comparison of TMB between RNF43-MUT and RNF43-WT patients in MSKCC cohort, in-house cohort and 3Dcohort, respectively. D. Effects of *RNF43* status and immune cell infiltration. E. Boxplot of differentially expressed immune-related genes in RNF43-MUT and RNF43-WT groups. F. Boxplot of differentially expressed HLA family genes in RNF43-MUT and RNF43-WT groups. G. GSEA reveals prominent enrichment of signatures related to antigen processing and presentation in CRC patients with *RNF43* mutation. *P < 0.05, **P < 0.01, ***P < 0.001. TMB: tumor mutational burden.

gest that RNF43-MUT may be a potential positive predictor in CRC patients treated with ICBs.

CRC is a leading cause of death. Chemotherapy with or without targeted therapy has been the standard of care for CRC patients for three decades. Although immunotherapy has made great progress, it is only effective in a small number of MSI-H cancer cases, which account for only 5% of metastatic CRC (mCRC) cases [20]. *RNF43* mutations are not uncommon in tumors, particularly colon cancer [21], accounting for approximately 19.3% and 36.7% of the MSKCC cohort and in-house cohort. This suggests that findings related to RNF43-MUT could be applicable to more patients than other rare mutations. Unlike PD-L1 expression or TMB, RNF43-MUT can be easily detected by NGS.

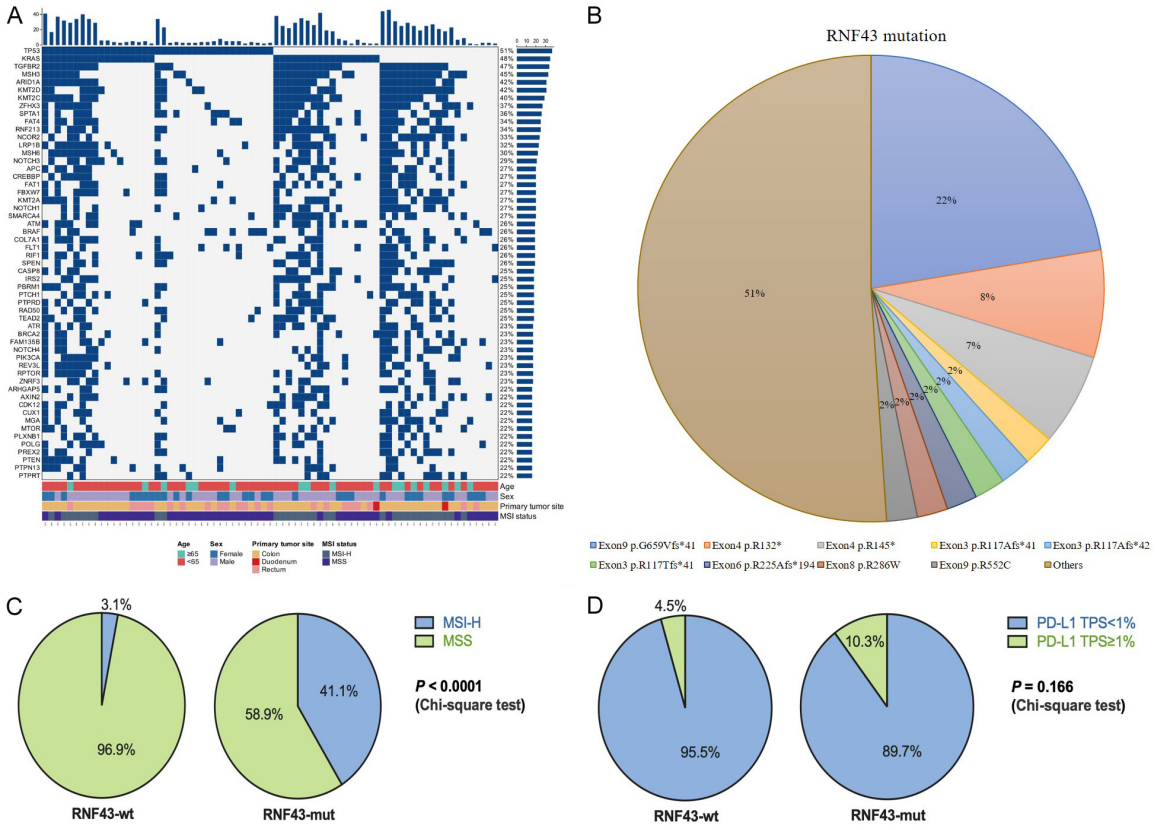

Figure 4. The genomic characteristics of CRC patients with *RNF43* mutations in 3Dcohort. A. The genomic landscape of RNF43-MUT CRC. B. *RNF43* high frequency mutation site. C, D. Comparison of MSI type and PD-L1 between RNF43-MUT (mutant-type) and RNF43-WT (wild-type) CRC patients. MSI: microsatellite instability.

Crucially, our study proposes RNF43-MUT as a potential biomarker for ICBs, highlighting an association between *RNF43* gene status and ICB response in a public cohort. Importantly, this association was further verified in our in-house CRC cohort, where patients with RNF43 mutations exhibited higher ORR, DCB, and improved prognosis.

RNF43 encodes an E3 ubiquitin ligase and negatively regulates Wnt signaling [21]. The mutation and down-regulation of *RNF43* may play a key role in the transformation of gastric adenoma to gastric carcinoma [22]. Similar findings were found in intestinal cancer, where Wnt signaling, activated by *RNF43* mutations during the tumorigenesis phase, promoted tumor growth and was associated with high recurrence rates in CRC patients [23]. Low expression of *RNF43* has been shown to be associated with a poorer prognosis in melanoma or clear cell renal cell carcinoma [24, 25]. In addition, PD-L1 has been reported to be related to *RNF43*. For example, in biliary tract cancers, *RNF43* mutations were predominantly enriched in the PD-L1 positive subgroup (7.1% vs. 1.4%, P = 0.003) [26]. Our study showed similar results, with significantly higher TMB for RNF43-MUT compared with RNF43-WT. This leads to the production of more neoantigens, which are processed by APCs and presented to T cells. The upregulation of MHC-related molecules facilitates this process. Concurrently, the expression of immune-related genes increased, with enrichment in the antigen processing and presentation pathway. This supports our belief that RNF43 mutations correlate with favorable clinical outcomes in ICBs.

To the best of our knowledge, this is the first time that the relationship between RNF43-MUT and ICBs has been explored in CRC. Our study still has some limitations, including those inherent in retrospective design. Analysis was based on the public cohort, which may have contributed to selection bias. However, our in-

dependent in-house CRC cohort serves as a robust complement to the analysis. Due to the limited number of patients, the relationship between RNF43-MUT and the efficacy of immunotherapy remains challenging for further exploration, but the overall efficacy of RNF43-MUT remains promising. These findings should be validated in larger cohorts. Additionally, the potential mechanisms by which *RNF43* mutations enhance the efficacy of immunotherapy were only explored by bioinformatics analysis. Our conclusion is only that *RNF43* mutation is related to an immune hot environment. The exact mechanism by which RNF43-MUT induces immune changes remains to be elucidated and deserves further study.

In conclusion, our results suggest that *RNF43* mutations correlate with improved OS through increased TMB and immune-related gene signatures in CRC patients treated with ICBs. *RNF43* mutations may be a crucial component of immunogenetics and should be considered as a therapeutic biomarker for immunotherapy.

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Disclosure of conflict of interest

None.

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Table S1. Baseline characteristics in our in-house cohort

Characteristics	RNF43-MUT (n = 22)	RNF43-WT (n = 38)	*P* value
Age, n (%)			
> 60	6 (27)	9 (24)	0.766
≤ 60	16 (73)	29 (76)	
Sex, n (%)			
Male	13 (59)	23 (61)	> 0.9999
Female	9 (41)	15 (39)	
Primary tumor site, n (%)			
Rectum	1 (5)	12 (32)	0.0346
Colorectal	20 (90)	23 (61)	
Others	1 (5)	3 (8)	
MSI status, n (%)			
MSI-H	13 (59)	12 (32)	0.0655
MSS	1 (5)	8 (21)	
NA	8 (36)	18 (47)	

RNF43-MUT: RNF43 mutation; RNF43-WT: RNF43 wile-type.

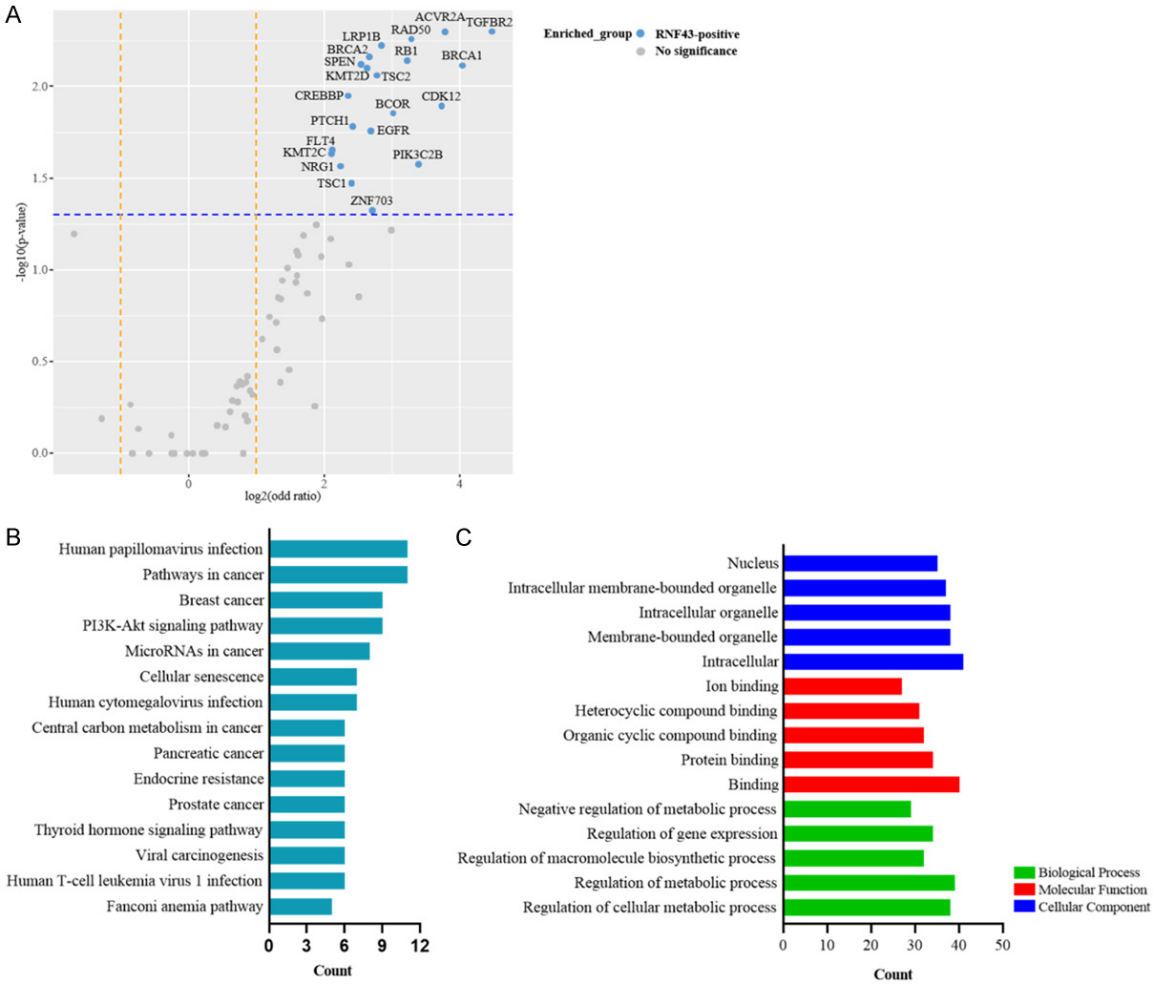

Figure S1. Differential expression (DE) analysis between *RNF43* mutation and *RNF43* wile-type in the hcohort. A. Different genes preference for *RNF43* mutations. B. The KEGG pathways analysis. C. The GO pathways analysis.

Table S2. Baseline characteristics in 3Dcohort

Characteristics	RNF43-MUT (n = 73)	RNF43-WT (n = 800)	*P* value
Age, n (%)			
≥ 60	17 (23.3%)	244 (30.5%)	0.198
< 60	56 (76.7%)	556 (69.5%)	
Sex, n (%)			
Male	43 (58.9%)	498 (62.3%)	0.573
Female	30 (41.1%)	302 (37.8%)	
Primary tumor site, n (%)			
Colorectal	49 (67.1%)	464 (58.0%)	0.269
Rectum	22 (30.1%)	293 (36.6%)	
Duodenum	2 (2.7%)	43 (5.4%)	
MSI status, n (%)			
MSI-H	30 (41.1%)	25 (3.1%)	< 0.0001
MSS	43 (58.9%)	775 (96.9%)	

RNF43-MUT: *RNF43* mutation; RNF43-WT: *RNF43* wile-type.

Table S3. *RNF43* high frequency mutation site

RNF43 mutation type	Frequency	Proportion (%)
Exon9 *p.G659Vfs*41*	21	22.3
Exon4 *p.R132**	7	7.4
Exon4 *p.R145**	6	6.4
Exon3 *p.R117Afs*41*	2	2.1
Exon3 *p.R117Afs*42*	2	2.1
Exon3 *p.R117Tfs*41*	2	2.1
Exon6 *p.R225Afs*194*	2	2.1
Exon8 *p.R286W*	2	2.1
Exon9 *p.R552C*	2	2.1
Others	48	51.1