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Clinical and molecular characteristics of *RNF43* mutations as promising prognostic biomarkers in colorectal cancer

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Zi-Yao Huang*, Lei Wen*, Liu-Fang Ye*, Yu-Ting Lu, William Pat Fong, Ren-Jing Zhang, Si-Xian Wu, Zhi-Gang Chen, Yan-Yu Cai, Rui-Hua Xu, Yu-Hong Li, Zi-Ming Du and De-Shen Wang

Correspondence to:
Yu-Hong Li
De-Shen Wang
Department of Medical Oncology, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou 510060, P. R. China

Research Unit of Precision Diagnosis and Treatment for Gastrointestinal Cancer, Chinese Academy of Medical Sciences, Guangzhou 510060, P. R. China
liyh@sysucc.org.cn
wangdsh@sysucc.org.cn

Zi-Ming Du
Department of Medical Oncology, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou 510060, P. R. China

Department of Molecular Diagnostics, Sun Yat-sen University Cancer Center, Guangzhou 510060, P. R. China
duzm1@sysucc.org.cn

Zi-Yao Huang
Lei Wen
Liu-Fang Ye
Yu-Ting Lu
William Pat Fong
Si-Xian Wu
Zhi-Gang Chen
Yan-Yu Cai
Rui-Hua Xu
Department of Medical Oncology, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou, P. R. China

Research Unit of Precision Diagnosis and Treatment for Gastrointestinal Cancer, Chinese Academy of Medical Sciences, Guangzhou, P. R. China

Abstract

Background: Transmembrane E3 ubiquitin ligase (*RNF43*) mutations are present in approximately 6–18% of colorectal cancers (CRC) and could enhance Wnt/β-catenin signaling, which is emerging as a promising therapeutic target. This study aims to investigate the clinical and molecular characteristics and potential heterogeneity of *RNF43*-mutant CRC.

Methods: A total of 78 patients with *RNF43*-mutant CRC were enrolled from July 2013 to November 2022. Demographic data, clinical characteristics, treatment regimens used, and survival outcomes were collected and analyzed.

Results: Our study uncovered that patients with *RNF43* mutations in the N-terminal domain (NTD; $n = 50$) exhibited shorter overall survival (OS; median months, 50.80 *versus* not reached; $p = 0.043$) compared to those in the C-terminal domain (CTD; $n = 17$). Most *RNF43* mutations in NTD had positive primary lymph node status, low tumor mutation burden (TMB-L), and correlated with proficient mismatch repair (pMMR)/microsatellite stable (MSS) status. By contrast, *RNF43* mutations in CTD were significantly enriched in deficient MMR (dMMR)/microsatellite instability (MSI-H) tumors with high TMB (TMB-H). N-terminal *RNF43*-mutated tumors harbored a hotspot variant (*RNF43 R117fs*), which independently predicted a significantly worse outcome in pMMR/MSS CRC with a median OS of 18.9 months. Patients with *RNF43* mutations and the *BRAF V600E* alterations demonstrated sensitivity to BRAF/EGFR inhibitors. Moreover, we observed that pMMR/MSS patients with *RNF43 R117fs* mutation had a higher incidence of stage IV, ⩾2 metastatic sites, low TMB, and none of them received PD-1/PD-L1 inhibitor therapy.

Conclusion: Our findings provide the first evidence that *RNF43* mutations in NTD and the *R117fs* variant correlate with a poorer prognosis in CRC patients, providing strategies for Wnt-targeted therapy to improve clinical efficacy.

Keywords: colorectal cancer, predictive biomarker, prognosis, *R117fs* variant, *RNF43* mutations

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Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide and the second leading cause of cancer-related mortality.[1] Previous studies suggest that most CRCs arise either *via* the adenoma-carcinoma sequence or the serrated neoplasia pathway.[2,3] Wnt/β-catenin signaling, the conventional pathway initiated by alterations in Wnt ligand-dependent genes (*RNF43*/*ZNRF3*/*RSPO*) or ligand-independent genes (*APC*), drives colorectal carcinogenesis.[4–6] *RNF43*, a transmembrane E3 ubiquitin ligase, acts as a feedback suppressor of the Wnt pathway by promoting the degradation of Frizzled (FZD) receptors[7] *via* ubiquitination.

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Ren-Jing Zhang
Department of Medical Oncology, State Key Laboratory of Oncology in South China, Guangdong Provincial Clinical Research Center for Cancer, Sun Yat-sen University Cancer Center, Guangzhou, P. R. China

Department of Molecular Diagnostics, Sun Yat-sen University Cancer Center, Guangzhou, P. R. China

*These authors contributed equally as co-first authors

RNF43 is composed of two specific functional domains, the N-terminal domain (NTD) and the C-terminal domain (CTD). The NTD is mainly responsible for the inhibition of Wnt/β-catenin signaling and consists of an extracellular protease-associated (PA) domain that interacts with R-spondins (RSPO) or FZD, a single-pass transmembrane (TM) domain, and an intracellular RING finger (RING) domain that has ubiquitination functions.[7–10] The CTD of *RNF43* contains the Disheveled-2 (DVL2) binding region (DIR), which is required for *RNF43*-mediated ubiquitination by binding with FZD receptors.[7,9–13] Nevertheless, the impact of DIR on oncogenesis remains highly controversial. There are two main hotspot subtypes of *RNF43* mutations, namely *G659fs* and *R117fs*. In malignancies, mutations in the NTD of the *RNF43* protein usually result in loss of function due to truncating events such as frameshift indels and nonsense mutations. One such variant is *R117fs*, which compromises the negative feedback regulation of the Wnt/β-catenin signaling pathway, rendering cancer cells susceptible to inhibition by Wnt ligands.[7,12,14]

Previously, *RNF43* mutations have been identified in several cancer types, including CRC, ovarian cancer, pancreatic cancer, and gastric cancer.[15–17] Approximately 6–18% of CRC patients harbor *RNF43* mutations,[17] which mainly served as a late event in the progression from serrated adenoma to malignancy.[3,18] Studies investigating the impact of *RNF43* mutations in CRC have yielded conflicting results. Some studies have reported that *RNF43* mutations are associated with poor outcomes and higher recurrence rates regardless of the MSI status, while others believe that *RNF43* mutant tumors are associated with prolonged survival[19–22]; therefore, the prognostic value of *RNF43* remains to be determined. Interestingly, tumors with *RNF43* mutations frequently exhibit a high frequency of *BRAF V600E* mutation, and the co-occurrence of these mutations is associated with worse survival outcomes. This subgroup of patients might benefit from anti-BRAF/EGFR therapy.[23–26] Nevertheless, it is worth noting that a phase Ib/II study of WNT974 + encorafenib + cetuximab in patients with *BRAF V600E* mutant metastatic CRC was recently discontinued due to bone-related toxicities.[27] Mutations in the NTD of *RNF43* significantly enhance Wnt/β-catenin signaling and can be inhibited by Porcupine inhibitors. By contrast, mutations in the CTD, such as the *G659fs* variant, are commonly found in microsatellite instability (MSI) tumors and may respond to anti-PD-1/PD-L1 therapy.[28]

Taken together, the above findings highlight the potential heterogeneity of *RNF43* mutant tumors. Given that the prognostic implications of distinct genetic subgroups within *RNF43* mutated CRC have not been elucidated, this study primarily aims to investigate the clinical, molecular, and potential prognostic characteristics of *RNF43*-positive CRC patients. Collectively, our findings will provide crucial insights into the role of *RNF43* in Wnt/β-catenin signaling activation, allowing for the stratification of patients with distinct prognoses and the identification of potential therapeutic strategies.

Materials and methods

Patient and sample collection

We evaluated 78 patients with *RNF43*-mutant CRC between July 2013 and November 2022 at Sun Yat-sen University Cancer Center (SYSUCC, Guangzhou, China). Molecular alterations were detected in tumor tissue using Sanger sequencing or in tumor or plasma samples using next-generation sequencing (NGS). Variables including demographics (age, gender), clinical characteristics [histology pathologic differentiation, primary tumor location, Tumor-Node-Metastasis (TNM) stage, primary lymph node status, number of metastatic sites], treatment features (PD-1/PD-L1 inhibitors), and survival were also collected. Immunohistochemistry (IHC) was used to classify mismatch repair (MMR) as proficient MMR (pMMR) or deficient MMR (dMMR) by assessing four MMR proteins (MLH1, MSH2, MSH6, and PMS2). Microsatellite instability (MSI) status was determined *via* NGS in tumor tissue and classified as high MSI (MSI-H), microsatellite stable (MSS), and low MSI (MSI-L). Tumor mutational burden-high (TMB-H) was defined as the presence of at least 10 mutations per megabase (Mb),[29] and overall survival (OS) was defined as the time from disease diagnosis to death or end of follow-up.

Analysis of molecular features

We comparatively analyzed the genomic landscapes of *RNF43*-altered metastatic colorectal cancer (mCRC) patients from the Memorial Sloan Kettering Cancer Center (MSKCC,

Cancer Cell 2018) using the cBioPortal database (https://www.cbioportal. org).

We stratified truncating and damaging missense mutations to NTD and CTD using codon 313 as a cutoff to demarcate the RING region. The in *silico* analyses of the missense variants utilized Polymorphism Phenotyping ver. 2 (PolyPhen-2, http://genetics. bwh. harvard. edu/pph2) to predict the functional effects of *RNF43* protein.[30] The PolyPhen-2 software categorizes the investigated mutations as probably damaging (probability score $\geqslant 0.85$), possibly damaging (probability score between 0.16 and 0.85), or benign (probability score $\leqslant 0.15$).

Statistical analysis

The chi-square test or Fisher's exact test was used to identify the differences in clinical characteristics, treatment features, and genomic alterations between groups. OS was examined using the Kaplan–Meier method and compared using the log-rank test. Univariate and multivariate Cox proportional hazard regression models were used to estimate the individual hazard ratio (HR). The HR with a 95% confidence interval (CI) was measured to estimate the hazard risk of individual factors. All statistical results were considered significant if the p value <0.05. Data were analyzed using SPSS software (version 24. 0, Armonk, NY:IBM Corp) and R software (version 4. 2. 0, R Foundation for Statistical Computing, https://www.r-project.org/, Vienna, Austria).

Results

Patient characteristics

Demographic and clinicopathological characteristics of the enrolled patients are summarized in Table 1. Of the 78 patients included, 49 (62.8%) were males and the median age at diagnosis was 49 years. Most patients had adenocarcinomas (71.8%) that were well or moderately differentiated (39.7%). Right-sided tumors were detected in 36 (46.2%) patients, and 61 (78.2%) patients had positive primary lymph node status. A total of 18 patients (23.1%) had more than one metastatic site, 41 (52.6%) were TNM stage IV, 30 (38.5%) had dMMR/MSI-H tumors, and 32 (41.0%) had a high tumor mutation burden (TMB-H). Meanwhile, 23 patients (29.5%) received immunotherapy and 7 patients were treated with a combination of vemurafenib, cetuximab, and irinotecan (VIC regimen). In terms of first-line regimen, 23 patients received Bevacizumab plus systemic chemotherapy, 8 patients were treated with cetuximab plus systemic chemotherapy, and 15 received standard chemotherapy only. In addition, we observed that among the included patients, 30 had concurrent KRAS mutations, 13 had BRAF V600E mutations, 24 had APC mutations, and 53 had TP53 mutations. Based on the estimation of mutation frequency, 15 patients exhibited *RNF43 Gly659fs* alterations, 10 had *RNF43 Arg117fs* alterations, and 3 patients presented with both.

The somatic mutational landscape in patients with RNF43 mutated CRC

In *RNF43*-mutant tumors with dMMR/MSI-H status, the most commonly mutated genes were *MLL2* (80%), *ARID1A* (77%), *RAD50* (63%), *NOTCH3* (60%), and *ASXL1* (60%) [Figure 1(a)]. By contrast, *TP53* (83%), *KRAS* (29%), *SMAD4* (23%), *LRP1B* (21%), and *PIK3CA* (21%) mutations were most frequently observed in the pMMR/MSS group [Figure 1(b)]. DMMR/MSI-H tumors exhibited more abundant alterations, and the majority of *RNF43* mutations were multi-hit; meanwhile, pMMR/MSS tumors had more *RNF43* truncating mutations (Figure 1). Furthermore, we created a visualization depicting the co-mutation landscape involving *RNF43* in the MSKCC cohort. *RNF43* showed a high frequency of concurrent alterations with *MLL2* (68%), *ARID1A* (57%), *FAT1* (53%), *NOTCH3* (51%), and *SPEN* (51%) in the dMMR/MSI-H subgroup. However, in the pMMR/MSS subgroup, the most frequently co-occurring alterations with *RNF43* were *TP53* (77%), *BRAF* (48%), *KRAS* (32%), *ARID2* (26%), and *SMAD4* (26%), which is consistent with our findings (Supplemental Figure S1).

The most frequent *RNF43* mutation in our cohort was the *p.Gly659fs* variant in exon 9, which was observed in 18 out of 78 patients (23.08%). Meanwhile, the second most common mutation was the *p.Arg117fs* variant in exon 3, observed in 13 out of 78 patients (16.67%) (Supplemental Figure S2). All the *p.Gly659fs* variants were mutated in dMMR/MSI-H tumors. Moreover, the lollipop diagram illustrated that the majority of *RNF43* mutations were truncating events (74%, 58/78) with a tendency to be located in the

Table 1. Clinicopathological and molecular characteristics of 78 patients in *RNF43*-mutant CRC.

Characteristics	No. patients (%)
	Overall (*N* = 78)
Age	
Mean ± SD	49.28(±13.58)
Gender	
Male	49 (62.8%)
Female	29 (37.2%)
Vital status	
Death	26 (33.3%)
Alive	53 (66.7%)
OS	
Median, CI	57.40 (48.76–64.78)
Histology	
Adenocarcinoma	56 (71.8%)
Signet/mucinous	19 (24.4%)
Else	3 (3.8%)
Pathologic differentiation	
Well or moderate	31 (39.7%)
Poor	32 (41.0%)
Else	15 (19.2%)
Tumor location	
Left	41 (52.6%)
Right	36 (46.2%)
Multi-side	1 (1.3%)
Primary lymph node status	
Positive	61 (78.2%)
Negative	11 (14.1%)
Unknown	6 (7.7%)
Number of organs involved	
<2	59 (75.6%)
⩾2	18 (23.1%)
NA	1 (1.3%)
TNM stage	
I–III	29 (37.2%)
IV	41 (52.6%)
NA	8 (10.3%)
MMR/MSI	
dMMR/MSI-H	30 (38.5%)
pMMR/MSS	48 (61.5%)
TMB	
H	32 (41.0%)
L	44 (56.4%)
NA	2 (2.6%)
RNF43	
G659 mutant	15 (19.2%)
R117 mutant	10 (12.8%)
Else	53 (68.0%)
KRAS	
Mutant	30 (38.5%)
Wild type	48 (61.5%)
BRAF	
V600 mutant	13 (16.7%)
Else[a]	65 (83.3%)
APC	
Mutant	24 (30.8%)
Wild type	54 (69.2%)
TP53	
Mutant	53 (67.9%)
Wild type	25 (32.1%)
Lynch	
Yes	14 (17.9%)
No	64 (82.1%)
PD-1 during the disease	23 (29.5%)
VIC[b] during the disease	7 (9.0%)
First-line therapy	
Bevacizumab + chemotherapy	23 (29.5%)
Cetuximab + chemotherapy	8 (10.3%)
Chemotherapy	15 (19.2%)

[a]BRAF else subgroup including wild types and else mutations except *BRAF V600E*.
[b]VIC regimen involved a combination of vemurafenib, cetuximab, and irinotecan.
CRC, colorectal cancer; OS, overall survival; TMB-H, patients with high TMB; TMB-L, patients with low TMB.

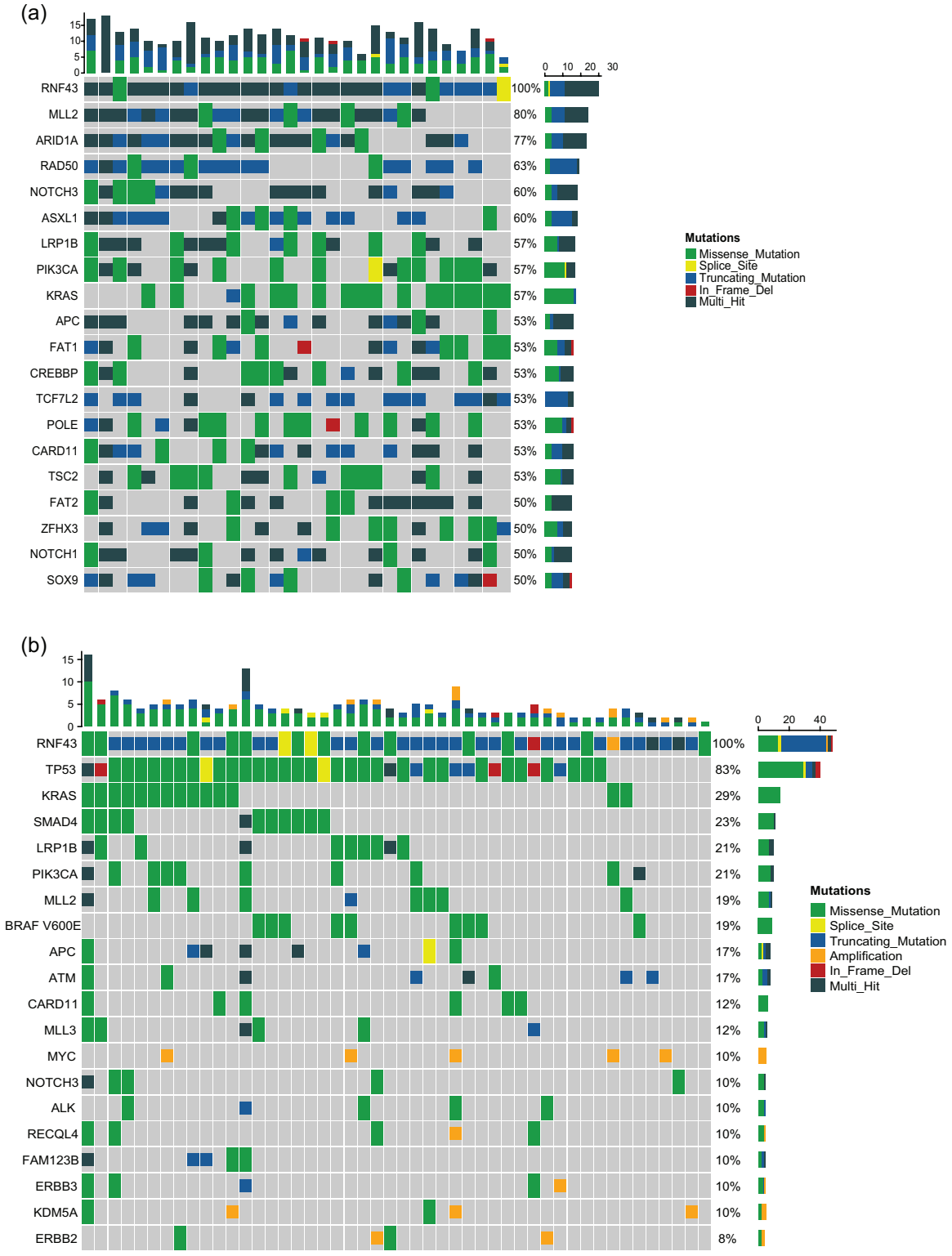

Figure 1. The mutational landscape shows the high-frequency genomic alterations detected in *RNF43*-mutant colorectal cancers with [(a), $n = 30$] dMMR/MSI-H and [(b), $n = 48$] pMMR/MSS. The colors of the bars are indicative of the type of mutation, with gray = wild type.

first half of the *RNF43* protein, while missense mutations were more evenly distributed throughout the protein (Supplemental Figure S2).

The prognostic profiles of RNF43 mutations across distinct protein domains

Truncating mutations and damaging missense mutations could cause the loss of function in *RNF43*. We categorized them into the NTD and CTD based on the protein structure at amino acid 313, with the NTD containing the PA, TM, and RING domains (Supplemental Figure S2).

Notably, alterations in NTD were associated with a worse prognosis for OS ($p = 0.043$; median OS, 50.80 months) compared to CTD [Figure 2(a)]. In addition, as shown in Figure 2 and Table 2, most of the tumors in NTD tended to have positive primary lymph node status (NTD *versus* CTD, 82.0% *versus* 52.9%; $p = 0.028$). However, patients with C-terminal alterations preferred to have mutations in dMMR/MSI-H tumors (NTD *versus* CTD, 32.0% *versus* 70.6%; $p = 0.009$) and a higher proportion of high tumor mutation burden (TMB-H) (NTD *versus* CTD, 32.0% *versus* 70.6%; $p = 0.004$). No statistically significant differences between CTD and NTD were observed in other clinicopathological characteristics.

The prognostic heterogeneity of RNF43 mutations in different subgroups

As illustrated in Supplemental Figure S2, the hotspot *RNF43* mutation, *p.Arg117fs* variant, was located in the N-terminal region of the protein. We classified the tumor samples into five molecular subtypes: dMMR/MSI, pMMR/MSS R117, pMMR/MSS BRAF VIC (a combination regimen of vemurafenib, cetuximab, and irinotecan), pMMR/MSS BRAF ST (standard chemotherapy), and pMMR/MSS ELSE.

With a total of 78 patients, the median OS was 57.40 months. Among *RNF43*-mutant patients with *BRAF V600E* alteration in the pMMR/MSS subgroup, those treated with VIC therapy demonstrated a significantly better prognosis ($p = 0.011$; median OS, 47.67 months), whose survival curves resembled those of the pMMR/MSS ELSE subgroup. Notably, we observed that the pMMR/MSS R117 subgroup exhibited a notably shorter overall survival ($p < 0.001$; median OS, 18.9 months), with its survival curve comparable to that of the pMMR/MSS BRAF ST subgroup [$p = 0.187$; Figure 2(b)].

The results of the univariate Cox regression analysis revealed that TNM-stage, number of organs involved, *APC* and *RNF43* subgroups were all significant prognostic factors for OS (Table 3). After adjusting for other clinicopathological factors, the number of organs involved and *RNF43* subtypes remained independent factors for OS. Most importantly, the pMMR/MSS BRAF VIC subgroup displayed a similar outcome to the pMMR/MSS ELSE subgroup (HR: 1.00; 95% CI, 0.29–3.55; $p = 0.992$). On the other hand, the presence of *RNF43 R117fs* mutation was associated with a significantly poorer prognosis in pMMR/MSS patients (HR: 9.31; 95% CI, 2.19–39.48; $p = 0.002$; Table 3).

Clinical and molecular characteristics of RNF43 mutations in different subgroups

To further explore the clinical and molecular characteristics of *RNF43 R117fs* mutation, we assigned patients into three groups. In summary, no statistical differences were observed among the dMMR/MSI-H, pMMR/MSS-R117, and pMMR/MSS-ELSE subgroups when stratified by demographics (age and gender), primary tumor characteristics (histology, pathology differentiation, tumor location, and primary lymph node status), and molecular features (*KRAS* and *BRAF V600E* mutations) as shown in Table 4.

Nevertheless, our analysis revealed that patients in the pMMR/MSS-R117 subgroup had a significantly higher rate of metastasis to different organs ($\geqslant 2$ organs involved) (71.4% *versus* 3.3%; $p < 0.001$), a higher proportion of tumors at TNM stages IV (85.7% *versus* 30.0%; $p = 0.006$), and a lower tumor mutational burden (100.0% *versus* 3.3%, $p < 0.001$) compared to the dMMR/MSI-H subgroup. In particular, we observed that *R117fs* variants were concurrent exclusivity with *APC* mutations (53.3% *versus* 0.0%; $p = 0.012$) in pMMR/MSS tumors compared to the dMMR/MSI-H subgroup (Table 4).

Discussion

In this retrospective study, we selected 78 *RNF43* mutant patients from a cohort of more than 1000 colorectal cancer (CRC) patients, making it one of the largest series of *RNF43*-mut CRCs reported to date. Our dataset suggests that *RNF43 R117fs* mutation is strongly associated with a poor survival outcome and could serve as an independent prognostic factor in patients with pMMR/MSS tumors.

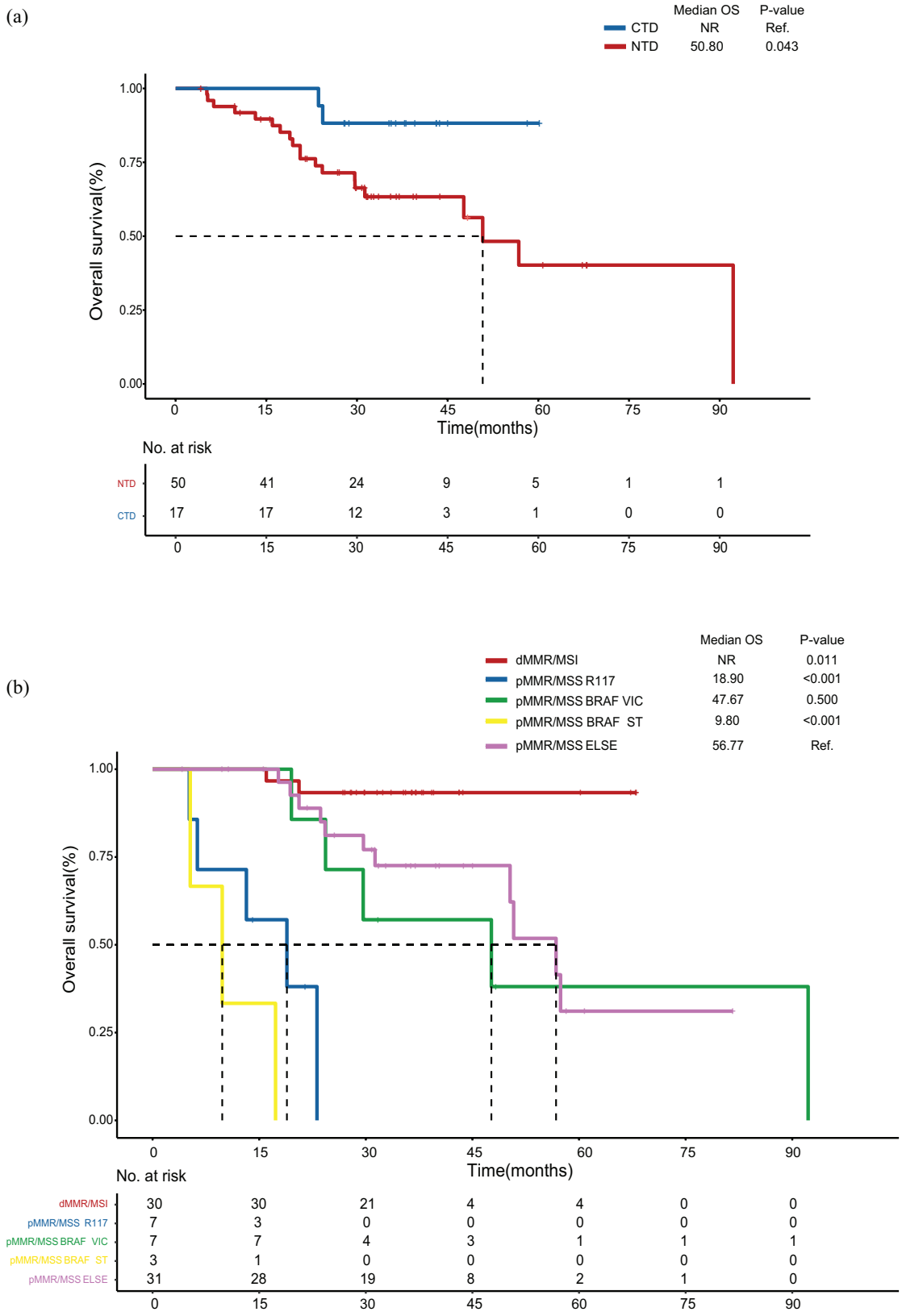

Figure 2. (a) Kaplan–Meier analysis of OS in CRC patients with truncating and damaging missense mutations between distinct domains of *RNF43* protein. (b) Kaplan–Meier analysis of OS in CRC patients with *RNF43* mutations in different subgroups. Cox models were used to obtain HRs with 95% CIs, and a two-sided log-rank test was used for statistical comparisons without adjustment for multiplicity. CRC, colorectal cancer; OS, overall survival; Ref., reference; VIC, a combination regimen of vemurafenib, cetuximab, and irinotecan; ST, standard chemotherapy.

Table 2. Differences in clinicopathological and molecular characteristics of *RNF43*-mutant CRC patients in distinct domains.

Characteristics	NTD (*N* = 50)	CTD (*N* = 17)	*p*-Value
Age	49.65 (±14. 94)	45.93 (±11. 22)	–
Gender			
Male	33 (66.0%)	8 (47.1%)	0.249
Female	17 (34.0%)	9 (52.9%)	
Histology			
Adenocarcinoma	36 (72.0%)	13 (76.5%)	0.888
Signet/mucinous	11 (22.0%)	4 (23.5%)	
Else	3 (6.0%)	0 (0.0%)	
Pathologic differentiation			
Well or moderate	20 (40.0%)	8 (47.1%)	0.375
Poor	19 (38.0%)	8 (47.1%)	
Else	11 (22.0%)	1 (5.9%)	
Tumor location			
Right	23 (46.0%)	8 (47.1%)	1.000
Left	26 (52.0%)	9 (52.9%)	
Else	1 (2.0%)	0 (0.0%)	
Primary lymph node status			
Positive	41 (82.0%)	9 (52.9%)	**0.028**
Negative	5 (10.0%)	6 (35.3%)	
Unknown	4 (8.0%)	2 (11.8%)	
Number of organs involved			
<2	36 (72.0%)	14 (82.4%)	0.651
$\geqslant 2$	13 (26.0%)	3 (17.6%)	
NA	1 (2.0%)	0 (0.0%)	
TNM stage			
I–III	17 (34.0%)	10 (58.8%)	0.088
IV	25 (50.0%)	7 (41.2%)	
NA	8 (16.0%)	0 (0.0%)	
MMR/MSI			
dMMR/MSI-H	16 (32.0%)	12 (70.6%)	**0.009**
pMMR/MSS	34 (68.0%)	5 (29.4%)	

(Continued)

Table 2. (Continued)

Characteristics	NTD (*N* = 50)	CTD (*N* = 17)	*p*-Value
TMB			
H	16 (32.0%)	12 (70.6%)	**0.004**
L	33 (66.0%)	4 (23.5%)	
NA	1 (2.0%)	1 (5.9%)	
KRAS			
Mutant	16 (32.0%)	8 (47.1%)	0.380
Wild type	34 (68.0%)	9 (52.9%)	
BRAF			
V600 mutant	11 (22.0%)	1 (5.9%)	0.270
Else[a]	39 (78.0%)	16 (94.1%)	
APC			
Mutant	12 (24.0%)	8 (47.1%)	0.123
Wild type	38 (76.0%)	9 (52.9%)	
TP53			
Mutant	35 (70.0%)	9 (52.9%)	0.243
Wild type	15 (30.0%)	8 (47.1%)	
PD-1 during the disease			
Yes	13 (26.0%)	7 (41.2%)	0.357
No	37 (74.0%)	10 (58.8%)	

Bold text indicates significance at $p < 0.05$.
[a]BRAF else subgroup including wild types and else mutations except *BRAF V600E*.
CRC, colorectal cancer; OS, overall survival; TMB-H, patients with high TMB; TMB-L, patients with low TMB.

Table 3. Univariate and multivariate Cox proportional hazards analysis of OS for patients in *RNF43*-mutant CRC.

Subgroup	Univariate Cox analysis			Multivariate Cox analysis		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Histology (adeno *versus* signet/mucinous)	1.002	0.417–2.404	0.997			
Differentiation (well or moderate *versus* poor)	1.196	0.514–2.784	0. 677			
TNM (I–III *versus* IV)	2.821	1.106–7.194	**0.030**	2.579	0.194–34.349	0.473
Location (left *versus* right)	1.188	0.529–2.670	0.676			
Primary lymph node status (negative *versus* positive)	3.239	0.746–14.071	0. 117			
Number of organs involved (<2 *versus* ⩾2)	11.278	4.347–29.263	<**0.001**	3.906	1.137–13.424	**0.031**

(Continued)

Table 3. (Continued)

Subgroup		Univariate Cox analysis			Multivariate Cox analysis		
		HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
KRAS (mutant *versus* WT)		0.458	0.183–1.148	0.096			
APC (mutant *versus* WT)		0.169	0.040–0.717	**0.016**	0.545	0.107–2.786	0.466
TP53 (mutant *versus* WT)		1.120	0.459–2.734	0.804			
Group	dMMR/MSI	0.176	0.039–0.799	**0.024**	0.062	0.006–0.694	**0.024**
	pMMR/MSS R117	12.488	3.515–44.367	**<0.001**	9.306	2.194–39.482	**0.002**
	pMMR/MSS BRAF VIC	1.483	0.470–4.683	0.502	1.006	0. 286–3.546	**0.992**
	pMMR/MSS BRAF ST	46.839	8.498–258.157	**<0.001**	28.129	3.795–208.496	**0.001**
	pMMR/MSS ELSE[a]	Ref.	Ref.	Ref.	Ref.	Ref.	Ref.
PD-1 (yes *versus* no)		0.265	0.079–0.887	**0.031**	2.357	0.476–11.676	0.294

Bold text indicates significance at $p < 0.05$.
[a]pMMR/MSS ELSE subgroup including patients treated with standard chemotherapy without *RNF43 R117* mutation and *BRAF V600E* mutation.
CRC, colorectal cancer; OS, overall survival; TMB-H, patients with high TMB; TMB-L, patients with low TMB; WT, wild type; VIC, target therapy (VIC regimen, a combination of vemurafenib, cetuximab, and irinotecan); ST, standard chemotherapy.

Table 4. Differences of clinicopathological and molecular characteristics between subgroups in *RNF43*-mutant CRC patients. p_1 value, dMMR/MSI *versus* pMMR/MSS R117; p_2 value, pMMR/MSS R117 *versus* pMMR/MSS else.

Characteristics	No. Patients (%)					
	dMMR/MSI (*N* = 30)	pMMR/MSSR117 (*N* = 7)	pMMR/MSSelse[b] (*N* = 41)	*p*-Value	p_1-Value	p_2-Value
Age	48.53 (±14.45)	49.29 (±14.23)	49.83 (±13.14)	–	–	–
Gender						
Male	21 (70.0%)	3 (42.9%)	25 (61.0%)	0.345	0td .213	0.429
Female	9 (30.0%)	4 (57.1%)	16 (39.0%)			
Histology						
Adenocarcinoma	20 (66.7%)	4 (57.1%)	32 (78.0%)	0.478	0.728	0.289
Signet/mucinous	9 (30.0%)	3 (42.9%)	7 (17.1%)			
Else	1 (3.3%)	0 (0.0%)	2 (4.9%)			
Pathologic differentiation						
Well or moderate	12 (40.0%)	3 (42.9%)	16 (39.0%)	0.927	0.871	0.668
Poor	12 (40.0%)	2 (28. 6%)	18 (43.9%)			
Else	6 (20.0%)	2 (28.6%)	7 (17.1%)			
Tumor location						
Right	13 (43.3%)	5 (71.4%)	18 (43.9%)	0.408	0.515	0.237
Left	16 (53.3%)	2 (28.6%)	23 (56.1%)			
Else	1 (3.3%)	0 (0.0%)	(0.0%)			

(Continued)

Table 4. (Continued)

Characteristics	No. Patients (%)					
	dMMR/MSI (*N* = 30)	pMMR/MSSR117 (*N* = 7)	pMMR/MSSelse[b] (*N* = 41)	*p*-Value	p_1-Value	p_2-Value
Primary lymph node status						
Positive	23 (76.7%)	7 (100.0%)	31 (75.6%)	0.850	0.769	0.738
Negative	4 (13.3%)	0 (0.0%)	7 (17.1%)			
Unknown	3 (10.0%)	0 (0.0%)	3 (7.3%)			
Number of organs involved						
<2	28 (93.3%)	2 (28.6%)	29 (70.7%)	**<0.001**	**<0.001**	0.080
⩾2	1 (3.3%)	5 (71.4%)	12 (29.3%)			
NA	1 (3.3%)	0 (0.0%)	0 (0.0%)			
TNM stage						
I–III	19 (63.3%)	0 (0.0%)	10 (24.4%)	**0.001**	**0.006**	0.458
IV	9 (30.0%)	6 (85.7%)	26 (63.4%)			
NA	2 (6.7%)	1 (14.3%)	5 (12.2%)			
TMB						
H	29 (96.7%)	0 (0.0%)	3 (7.3%)	**<0.001**	**<0.001**	1.000
L	1 (3.3%)	7 (100.0%)	36 (87.8%)			
NA	0 (0.0%)	0 (0.0%)	2 (4.9%)			
KRAS						
Mutant	16 (53.3%)	2 (28.6%)	12 (29.3%)	0.114	0.405	1.000
Wild type	14 (46.7%)	5 (71.4%)	29 (70.7%)			
BRAF						
V600 Mutant	2 (6.7%)	1 (14.3%)	10 (24.4%)	0.158	0.477	1.000
Else[a]	28 (93.3%)	6 (85.7%)	31 (75.6%)			
APC						
Mutant	16 (53.3%)	0 (0.0%)	8 (19.5%)	**0.002**	**0.012**	0.583
Wild type	14 (46.7%)	7 (100.0%)	33 (80.5%)			
TP53						
Mutant	14 (46.7%)	6 (85.7%)	33 (80.5%)	**0.006**	0.097	1.000
Wild type	16 (53.3%)	1 (14.3%)	8 (19.5%)			
Lynch						
Yes	14 (46.7%)	0 (0.0%)	0 (0.0%)	**<0.001**	**0.031**	–
No	17 (53.3%)	7 (100.0%)	41 (100.0%)			
PD-1						
Yes	19 (63.3%)	0 (0%)	4 (9.8%)	**<0.001**	**0.003**	1.000
No	11 (36.7%)	7 (100%)	37 (90.2%)			

Bold text indicates significance at $p < 0.05$.
[a]BRAF else subgroup including wild types and else mutations except *BRAF V600E*.
[b]pMMR/MSS else including patients without dMMR/MSI status and *RNF43 R117fs* mutation.
CRC, colorectal cancer; OS, overall survival; TMB-H, patients with high TMB; TMB-L, patients with low TMB; WT, wild type.

With a median age at diagnosis of 49 years, *RNF43*-mutant patients tend to be younger than the majority of CRC cases, which needed more concerns. The nature of the interaction between *RNF43* and Frizzled (FZD) receptors, specifically whether it occurs *via* the DVL-interaction region (DIR) or direct binding with the PA domain, remains a subject of debate. Besides, it has been suggested that some truncating mutations in the DIR domain may confer gain-of-function properties.[7,8,10,12,13] Hence, we opted to utilize the end of the RING region as a cutoff point to examine the impact of truncating and deleterious missense mutations, which was in line with previous investigations.[7–10]

Recent studies have shown that N-terminal truncating mutations of *RNF43* are more efficient in enhancing Wnt/β-catenin (canonical WNT pathway) signaling activity compared to C-terminal mutations, with the majority of these mutations being loss of function.[13,18,31] Importantly, our data suggest that the C-terminal region (CTD) of *RNF43* mutations is associated with a better OS, with most patients having dMMR/MSI-H and TMB-H, which could partly explain the better prognosis. Similar to previous reports, we observed that the most frequent mutations in our study were *Gly659fs* and *Arg117fs*, which were located in the C-terminal and N-terminal regions, respectively.[17] All *Gly659fs* mutations observed in our study were identified in dMMR/MSI-H tumors; moreover, the majority of these patients received immune checkpoint inhibitor (ICI) therapy and achieved a more favorable clinical outcome. While the *RNF43 R117fs* mutation was predominantly observed in pMMR/MSS tumors, suggesting its potential as a biomarker in pMMR/MSS patients. Previous studies by Elez *et al*. and our research have both indicated that *RNF43*-mutant tumors co-occurring with *BRAF V600E* alterations exhibit sensitivity to anti-BRAF/EGFR therapy.[25,26] As a result, we categorized patients into five subgroups: dMMR/MSI, pMMR/MSS R117, pMMR/MSS BRAF VIC (a combination regimen of vemurafenib, cetuximab, and irinotecan), pMMR/MSS BRAF ST (standard chemotherapy), and pMMR/MSS ELSE. Interestingly, *BRAF V600E* mutant patients who received the VIC regimen demonstrated extended OS in contrast to previous reports,[32,33] suggesting that *RNF43* mutation accompanied by *BRAF V600E* alteration represents a distinct subtype with predictive value for BRAF/EGFR inhibitor treatments. Importantly and innovatively, patients belonging to the pMMR/MSS R117 subtype exhibited a much poorer outcome, which was also found to be an independent prognostic factor. The observed difference in outcome between patients belonging to the pMMR/MSS R117 subtype and other subtypes could potentially be attributed to several factors, including the presence of more than two metastatic sites, a higher TNM stage, lower tumor mutational burden, and a lower likelihood of receiving ICI therapy.[29]

In agreement with our findings, a previous study found a significant difference in the location of *RNF43* mutations between MSI and MSS cancers, with the *RNF43 G659fs* mutation being frequently associated with colorectal tumors having an MSI phenotype and a favorable prognosis.[17] *RNF43 G659fs* mutation has been reported to have equal activity with wild type, which indicates that it might be a passenger mutation or a secondary mutation effect triggered by MSI status.[13,34] On the other hand, the characteristics of *RNF43 R117fs* mutation, which has been reported to positively regulate Wnt signaling, are still largely unknown.[12] Li *et al*. previously uncovered that among a panel of *RNF43*-mutant CRC cell lines, only HCT116, which carries a homozygous *R117fs* mutation, exhibited reduced pDVL2 levels and a consistent growth inhibition following Wnt-secretion blockade.[13] Numerous studies have highlighted the importance of *RNF43* in immune modulation, with implications for ICI therapy response.[22,35] Notably, our previous work demonstrated the predictive value of *RNF43* mutations in anti-PD-1/PD-L1 combination therapy for *BRAF V600E* mCRC patients.[25]

Co-mutations in *APC, KRAS, TP53*, and *SMAD4* are frequently observed in MSS-CRCs, while MSI-H CRCs harbor a higher frequency of *BRAF* and *RNF43* alterations.[36] It has been observed that *RNF43* mutations are exclusively associated with *APC* mutations and frequently co-occur with *BRAF V600E* mutations.[23] Likewise, all the tumors in our pMMR/MSS R117 group exhibited exclusivity with *APC* mutation. Tumorigenesis is a complex process that involves multiple factors, including genetic mutations in various pathways. Studies by Bert Vogelstein *et al*. and Yaeger *et al*. have demonstrated a potential trend in which right-sided colorectal tumors are associated with MSI-H status, *BRAF-RNF43* mutations, and poor prognosis, while left-sided tumors are linked to the MSS phenotype, *APC-KRAS* mutations, and better outcomes.[2,36–38] Moreover, Vogelstein *et al*. also surmise that *APC* mutations may serve as an early

rate-limiting event in the conventional pathway, while *RNF43* mutations are thought to occur in the later stages of the serrated pathway.[18,23,39,40] These studies suggest that the development of CRC may involve different mechanisms of activation for the WNT and RAS-RAF-MEK-ERK signaling pathways.[24,41] Both Elez *et al.* and our previous studies have demonstrated that *RNF43* mutations can improve the prognosis of patients with *BRAF V600E* mutant mCRC who are treated with anti-BRAF/EGFR therapy, providing further evidence for the co-activation of the WNT and MAPK signaling pathways.[25,26] Other studies have shown that *RNF43* mutations can downregulate P53 expression and promote tumorigenesis through a multi-step process involving the WNT-RAS-P53 signaling axis.[8,20] In addition, *RNF43 G659fs* mutation can increase PI3K signaling by promoting p85 ubiquitination.[28] Taken together, these findings suggest the existence of a potential cross-talk between the WNT/MAPK/TGF-β/PI3K/P53 pathway, warranting further investigation.

The activity of N-terminal alterations, specifically the *R117fs* mutation, appears to be reliant on the activation of WNT signaling, which can be targeted using blocking antibodies.[9,13,31] Porcupine inhibitors (PORCi) have been shown to have anti-tumor effects by inhibiting the secretion of Wnt-ligands, and *RNF43* loss-of-function mutations have been observed to be sensitive to PORCi treatment.[14,42] Moreover, blocking the WNT signaling pathway has been shown to activate the immune system by stimulating T cells and dendritic cells.[43,44] Therefore, targeting *RNF43* mutations may provide a viable strategy for anti-cancer drugs, such as LGK974 and ETC159, either alone or in combination with ICIs (ClinicalTrials.gov Identifier: NCT01351103).[42,45,46]

Nevertheless, the current study has a limited sample size, highlighting the need for further research using a larger cohort that also includes *RNF43* wild type. Moreover, the underlying mechanisms of *RNF43* mutations on tumor progression and therapy response are complex and require further elucidation. Therefore, additional investigations are necessary to fully understand the implications of *RNF43* mutations in CRC.

In conclusion, our study sheds light on the prognostic differences between the genetic subgroups of *RNF43* mutations in CRC. Our analysis provides comprehensive insights into *RNF43*-mutated CRC, including its association with clinical, molecular, and prognostic features. We verified that *RNF43* got a predictive value in response to BRAF/EGFR inhibitors in *BRAF V600E* tumors. Particularly, we found that the *RNF43 R117fs* mutation was associated with a poorer prognosis and could serve as a potential biomarker in *RNF43*-mutant CRC. Importantly, our findings could be utilized to stratify patients with CRC and guide treatment decisions.

Declarations

Ethics approval and consent to participate

Ethics approval of this retrospective study was obtained from the Ethical Committee of Sun Yat-sen University Cancer Center (code G2022-212-01).

Consent for publication

All authors have approved the submission of this manuscript for publication.

Author contributions

Zi-Yao Huang: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Lei Wen: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Liu-Fang Ye: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Yu-Ting Lu: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

William Pat Fong: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Ren-Jing Zhang: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Si-Xian Wu: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Zhi-Gang Chen: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Yan-Yu Cai: Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization; Writing – original draft; Writing – review & editing.

Rui-Hua Xu: Conceptualization; Funding acquisition; Project administration.

Yu-Hong Li: Conceptualization; Funding acquisition; Project administration.

Zi-Ming Du: Conceptualization; Funding acquisition; Project administration.

De-Shen Wang: Conceptualization; Funding acquisition; Project administration.

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Competing interests
The authors declare that there is no conflict of interest.

Availability of data and materials
Main data are shown in this article and additional data about this study can be obtained from the corresponding author on reasonable request.

ORCID iDs
Yu-Hong Li https://orcid.org/0000-0002-5710-9096

De-Shen Wang https://orcid.org/0000-0001-9657-4380

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