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Molecular profiling and identification of prognostic factors in Chinese patients with small bowel adenocarcinoma

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Abstract

Small bowel adenocarcinoma (SBA) is a rare malignancy with a poor prognosis and limited treatment options. Despite prior studies, molecular characterization of this disease is not well defined, and little is known regarding Chinese SBA patients. In this study, we conducted multigene next-generation sequencing and 16S ribosomal RNA gene sequencing on samples from 76 Chinese patients with surgically resected primary SBA. Compared with colorectal cancer and Western SBA cohorts, a distinctive genomic profile was revealed in Chinese SBA cohorts. According to the levels of clinical actionability to targetable alterations stratified by OncoKB system, 75% of patients harbored targetable alterations, of which *ERBB2*, *BRCA1/2*, and *C-KIT* mutations were the most common targets of highest-level actionable alterations. In DNA mismatch repair–proficient (pMMR) patients, significant associations between high tumor mutational burden and specific genetic alterations were identified. Moreover, *KRAS* mutations/*TP53* wild-type/nondisruptive mutations ($KRAS^{mut}/TP53^{wt/non\text{-}dis}$) were independently associated with an inferior recurrence-free survival (hazard ratio [HR] = 4.21, 95% confidence interval [CI] = 1.94-9.14, $P < .001$). The bacterial profile revealed *Proteobacteia*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Cyanobacteria* were the most common phyla in SBA. Furthermore, patients were clustered into three subgroups based on the relative abundance of bacterial phyla, and the distributions of the subgroups were significantly associated with the risk

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of recurrence stratified by *TP53* and *KRAS* mutations. In conclusion, these findings provided a comprehensive molecular basis for understanding SBA, which will be of great significance in improving the treatment strategies and clinical management of this population.

KEYWORDS
bacterial profile, Chinese, mutational landscape, recurrence-free survival, small bowel adenocarcinoma

1 | INTRODUCTION

Small bowel adenocarcinoma (SBA) is a rare malignancy accounting for only 1%-2% of gastrointestinal tumors.[1] Recently, the incidence of SBA is increasing, which has attracted the attention of the public.[2] Despite the close anatomical proximity between the small and large intestines, SBA is much less common than colorectal cancer (CRC).[2] The reason for this difference is currently unknown, and the limited knowledge of molecular characterization for SBA prevented us from explaining it.

As compared with CRC, there is a relative dearth of data on the pathogenesis and effective treatment in SBA. Due to the lack of prospective studies, there are no standard treatment strategies for SBA, and the position of novel targeted therapies still needs to be further defined. To date, the clinical management of SBA resembles that of CRC. However, besides the difference in prevalence between SBA and CRC, SBA prognosis has been shown to be worse than CRC prognosis.[3,4] Moreover, recent studies have reported that anti-EGFR therapy exhibited no clinical benefit in *RAS* wild-type (wt) SBA, which was in conflict with the CRC findings.[5] It is therefore clear that SBA had specific characterization, and the results derived from CRC studies cannot be presumed to guide clinical management decisions for SBA to an extent.

Within the past decade, the next-generation sequencing (NGS) technology has greatly contributed to the comprehensive analysis of genomic landscape, microbial community, and identification of molecular prognostic factors in various tumors.[6,7] However, compared with other types of solid tumors, only a few studies explored the genomic alterations of SBA, and most of them were performed in patients from Western countries.[8-10] Additionally, there were some limitations in these studies, including a small sample size, a lack of prognostic biomarkers, and a limited number of cancer-related genes to be assessed. Moreover, the role of bacteria in the development of human SBA has never been delineated. To date, little is known regarding the molecular characterization of Chinese SBA. Comprehensive molecular profiling of Chinese SBA is urgent to be performed, which may have great significance for prognosis and treatment.

Herein, multigene NGS and 16S ribosomal RNA gene sequencing in a cohort of Chinese patients with SBA were conducted. We aimed to elucidate the genomic and bacterial profiles, explore recurrence risk stratification biomarkers, and evaluate the clinical actionability of targetable alterations, which will contribute to promoting more effective clinical management of SBA.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

Tumor and matched normal tissue samples of 76 patients with surgically resected primary SBA were collected in this study. The patients were treated at multiple hospitals across China, including the Affiliated Sir Run Run Shaw Hospital of Zhejiang University School of Medicine, the Second Affiliated Hospital of Zhejiang University School of Medicine, and the Sixth Affiliated Hospital of Sun Yat-sen University between January 2016 and November 2018. All participants provided written informed consent before sample collection. This study was approved by the ethical committee of the Affiliated Sir Run Run Shaw Hospital of Zhejiang University School of Medicine.

2.2 | DNA extraction and library preparation

Tissue DNA in the present study was extracted using the QIAamp Genomic DNA kit (Qiagen), the quantification and quality control of which were evaluated by Qubit dsDNA HS assay kit (Thermo Fisher Scientific Inc) and Agilent 2100 BioAnalyzer (Agilent Technologies Inc). The sequencing libraries were generated according to the manufacturer's instructions (Illumina Inc).

2.3 | NGS

The libraries were enriched using an Acornmed panel targeting 808 cancer-related genes. The captured libraries were pooled and sequenced on the NovaSeq6000 System (Illumina Inc). Following the removal of the low-quality sequencing data, reads were aligned to the reference human genome (hg19) using BWA aligner v0.7.12. Base recalibration was performed using GATK v3.8. Single-nucleotide

variant (SNV) and small insertion or deletion (INDEL) callings were analyzed by MuTect2 v1.1.7. Copy number variant calling was conducted using CONTRA v2.0.8.

2.4 | Tumor mutational burden (TMB) estimation

TMB is defined as the number of somatic mutations per Mb, including SNV and INDEL. It is filtered according to the following rules: The variants included synonymous and nonsynonymous mutations. Variants with a mutant allele frequency (MAF) of >1% in the Exome Aggregation Consortium or 1000 Genomes databases were excluded.

2.5 | Bacterial 16S rRNA gene sequencing

Total DNA was extracted from samples, and the bacterial V3-V4 region of the 16S rRNA gene was amplified by Phusion High-Fidelity DNA polymerase (New England Biolabs). The analysis was conducted using Uparse software,[11] and sequences with ≥97% similarity were categorized into the same Operational Taxonomic Units (OTU). The relative abundance evaluation of the bacterial community was analyzed by the Chao 1 estimator.

2.6 | Statistical analysis

Categorical relationships were detected using Fisher's exact test. Differences in continuous variables between the groups were analyzed by the Mann-Whitney *U* test or one-way ANOVA test. The Kaplan-Meier method with log-rank test was used for univariate analyses, and a Cox proportional hazards regression model was used for multivariate analysis. A two-sided $P < .05$ was considered significant.

3 | RESULTS

3.1 | Clinical features and genomic profiles of Chinese SBA

Among the patients enrolled in this study, the overall median age at diagnosis was 61 years (range, 40-81 years), and 55.3% of the patients were male. The clinical characteristics of these patients are listed in Table 1. A total of 1011 somatic variants from 398 genes were identified. The most commonly mutated genes in our series of SBA were *TP53*, *APC*, *KRAS*, *SMAD4*, and *LRP1B* (Figure 1A). Mutation loci distributions of *KRAS* were further evaluated. *KRAS* mutations were mainly distributed in codon 12 (80.0%). The most common mutation in *KRAS* was G12D (32.0%), followed by *G12V* (24.0%) and *G13D* (12.0%) (Figure S1). Copy number variations of *EGFR*, *ERBB2*, *CDK12*, *FGFR1*, *FGFR3*, and *MDM2* were found (Table S1). Gene

TABLE 1 Clinical characteristics of enrolled patients with SBA

Characteristics	Number (%)
Age	
Age > 60 y	42 (55.3%)
Age ≤ 60 y	34 (44.7%)
Gender	
Male	42 (55.3%)
Female	34 (44.7%)
ECOG PS	
0-1	39 (51.3%)
≥2	37 (48.7%)
T stage	
1	3 (3.9%)
2	21 (27.7%)
3	26 (34.2%)
4	26 (34.2%)
N stage	
0	47 (61.9%)
1	27 (35.5%)
2	2 (2.6%)
M stage	
0	69 (90.8%)
1	7 (9.2%)
TNM stage	
I	20 (26.3%)
II	24 (31.6%)
III	25 (32.9%)
IV	7 (9.2%)
Lymphatic invasion	
Yes	22 (28.9%)
No	54 (71.1%)
Adjuvant chemotherapy	
Yes	28 (36.8%)
No	48 (63.1%)
Adjuvant chemotherapy regimen	
XELOX	7 (9.2%)
FOLFOX	5 (6.6%)
Tegafur	5 (6.6%)
Capecitabine	3 (3.9%)
Gemcitabine + Tegafur	2 (2.6%)
Gemcitabine + Capecitabine	1 (1.3%)
FOLFIRINOX	1 (1.3%)
SOX	1 (1.3%)
Unknown	3 (3.9%)
Tumor locations	
Duodenum	62 (81.6%)
Jejunum	9 (11.8%)

(Continues)

TABLE 1 (Continued)

Characteristics	Number (%)
Ileum	5 (6.6%)
MMR status	
dMMR	4 (5.3%)
pMMR	72 (94.7%)

Note: Unknown, patients underwent surgery in our hospital, but they received chemotherapy in other hospitals. We could not acquire the concrete chemotherapy regimen for these patients.

Abbreviations: dMMR, DNA mismatch repair–deficient; FOLFIRINOX, a combination of calcium folinate, fluorouracil, and irinotecan with oxaliplatin; FOLFOX, a combination of calcium folinate and fluorouracil with oxaliplatin; pMMR, DNA mismatch repair–proficient; PS, performance status; SOX, a combination of tegafur, gimeracil, oteracil potassium with oxaliplatin; XELOX, a combination of capecitabine with oxaliplatin.

rearrangements were identified in two patients including *NTRK3-ETV6* and *EWSR1-ETV1*. The affected signaling pathways were investigated based on the mutation data. The results showed that Wnt/β-catenin, MAPK, DNA damage repair (DDR), PI3K/AKT, epigenetic, and p53 signaling pathways were the most frequently altered pathways (Tables S2 and S3). Of note, the DDR pathway was remarkably altered in SBA. With the affected DDR pathway, the most commonly altered genes were *ATM*, *ATR*, and *POLD1* (Figure S2).

Somatic evolutionary associations of the driver mutations in *TP53*, *APC*, and *KRAS* were explored. Among all SBA patients, five patients carried mutations in all these three genes, and 35 patients harbored mutations in two genes whose MAFs demonstrated a significant linear relationship based on Pearson correlation analysis (Pearson $r = .753$, $P < .001$; Figure 1B), implying that mutations in these three genes generally co-occur as clonal events in the evolution of SBA. To further explore the mutational processes operating in SBA, mutational signatures were categorized by classifying the SNVs based on their trinucleotide mutational contexts.[12] Three independent mutational signatures were identified and matched known COSMIC signatures 1, 3, and 6, respectively (Figure 1C). These three signatures were then named based on the COSMIC signature nomenclature. Signature 1 is correlated with age and observed in various tumor types. Signature 3 is linked to homologous recombination deficiency (HRD) and has been found in breast, ovarian, and pancreatic cancers. Signature 6 is associated with DNA mismatch repair deficiency (MMRD) and most commonly occurs in colorectal and uterine cancers. Furthermore, the relationship between mutational signatures and tumor locations was further explored. Because of the small number of patients with jejunum and ileum adenocarcinomas (Table 1), we classified these patients into a single group, and then the mutational signatures between duodenum and jejunum/ileum adenocarcinomas were compared. Significant differences in the mutational signatures between the two groups were observed. Signature 1 and 3 were discovered in duodenum adenocarcinoma (Figure S3A), whereas signature 5 and 6 existed in jejunum/ileum adenocarcinoma (Figure S3B).

3.2 | Comparison of the genomic landscape between different cohorts

To investigate the specific genomic features related to Chinese SBA, the genomic landscape was compared between Chinese and Western SBA cohorts. However, somatic mutation data of SBA in the public databases are very limited currently. Therefore, mutation data from a study of a large series of Western SBA patients (n = 317) were used for the comparative analysis, although only 236 or 315 cancer-related genes were assessed in the study.[13] As compared with Western SBA cohorts, significantly fewer genomic alterations were observed in *TP53*, *KRAS*, *CDKN2A*, and *CDKN2B*, and significantly more mutations in *LRP1B* and *ARID2* were identified among Chinese SBA cohorts. A potential statistical difference was identified in *APC* and *FBXW7* between the two cohorts ($P = .091$ and $P = .097$, respectively). Although *PIK3CA* mutation rate in Chinese cohorts was lower than that in Western cohorts, no significant difference was observed (9.0% vs 16.1%, $P = .151$; Figure 2A). Furthermore, the comparison of the genomic landscape between Chinese SBA cohorts and CRC was conducted. As compared with the Cancer Genome Atlas (TCGA) CRC cohorts, marked differences were observed in the occurrence of *TP53*, *APC*, *ARID2*, *ERBB2*, *ELF3*, *PIK3CA*, and *ZFHX4* alterations. A potential statistical difference was observed in *SMAD4*, *PTPRS*, *NOTCH2*, and *TGFBR1* ($P = .075$, $P = .086$, $P = .052$, and $P = .054$, respectively; Figure 2B). Additionally, mutation data from a recent study[14] with a large series of Chinese CRC patients (n = 338) were utilized for the comparison of the genomic differences between Chinese SBA and Chinese CRC cohorts. Significant differences were identified in the frequencies of *TP53*, *APC*, *LRP1B*, *ERBB2*, *FAT1*, *NOTCH2*, *CTNNB1*, and *ELF3* mutations, and a potential statistical difference was identified in *PIK3CA* and *SMAD4* ($P = .080$ and $P = .068$, respectively) between the two groups (Figure 2C).

3.3 | TMB analysis in Chinese SBA

Accumulating evidence indicates that TMB acts as a potential biomarker to predict the outcome of PD-1/PD-L1 inhibitors in various types of cancer.[15] Tumor patients with high TMB may exhibit more neoantigens that could be recognized by the host immune system.[15] However, there is currently no study that comprehensively investigates the distributions of TMB in SBA, and whether TMB is associated with certain clinical or molecular features. In this study, the median TMB was 8.7 mutations/Mb (range 1.5-72.4 mutations/Mb) (Figure S4). The association between TMB and clinical characteristics was analyzed. No association was observed between TMB and gender or tumor site (Figure S5A,B), whereas TMB tended to be positively correlated with age (Pearson $r = .194$, $P = .094$; Figure S5C). Furthermore, the correlation between TMB and molecular features was evaluated. DNA mismatch repair–deficient (dMMR) tumors showed strikingly high TMB (Figure 3A). Increasing studies have reported that patients with DDR alterations exhibited high TMB in various cancer types.[16] However, the association between DDR

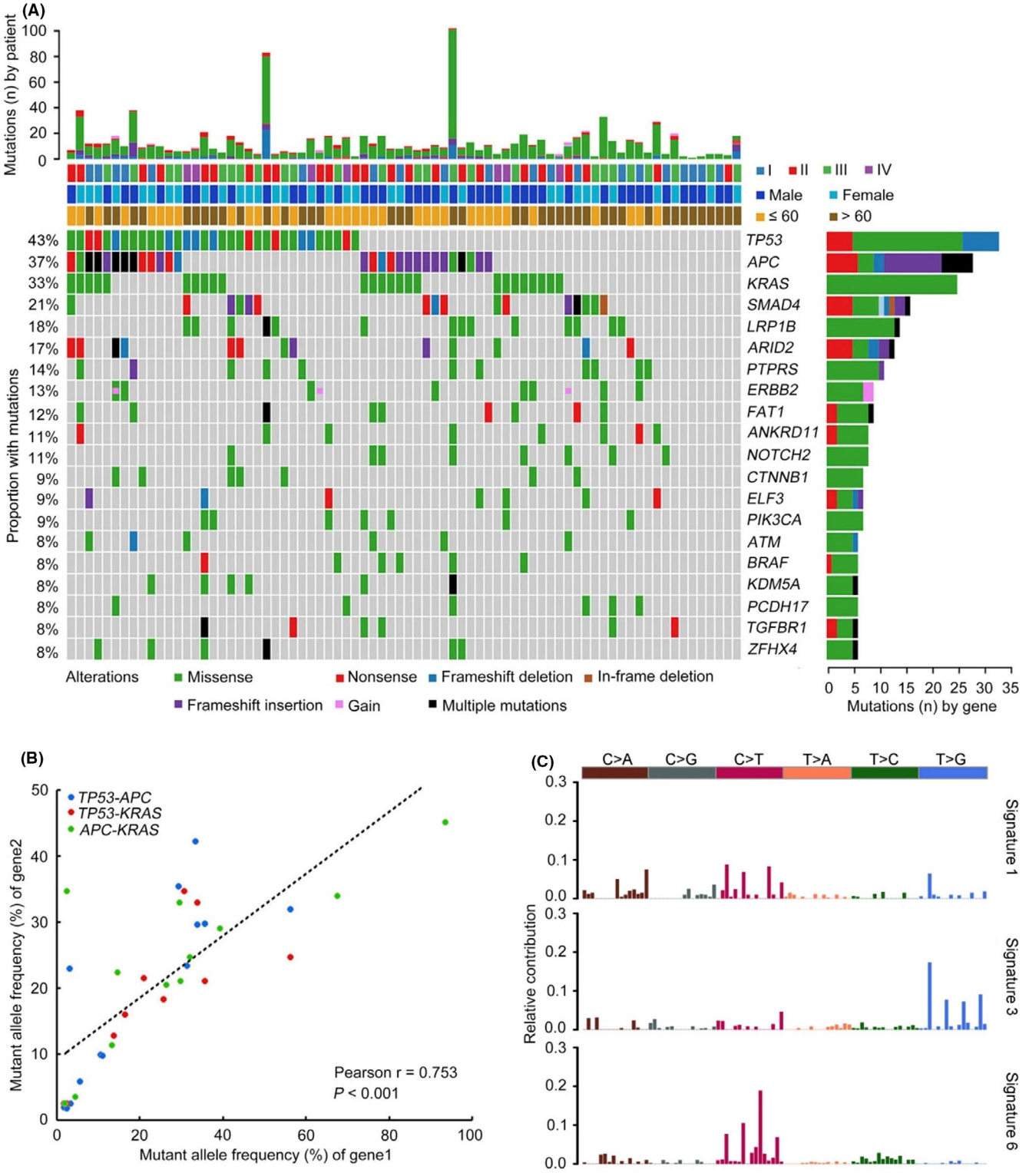

FIGURE 1 Mutational landscape of Chinese patients with small bowel adenocarcinoma (SBA). A, The somatic mutational profile of all cases. The upper panel shows the numbers of nonsynonymous single-nucleotide variants, small insertions or deletions, and copy number variants in each tumor. The central plot shows the key clinical parameters, below which the recurrently mutated genes for each case are exhibited. B, Correlation between mutant allele frequencies (MAFs) of *TP53*, *APC*, and *KRAS* in 35 patients with two mutated genes (*TP53-APC*, *TP53-KRAS*, *APC-KRAS*). C, Three independent mutational signatures identified in SBA

mutations and TMB in SBA was never investigated. Herein, no significant difference was observed in the distribution of TMB between tumors with and without DDR alterations (Figure S6). As shown in Figure S2, missense mutations were predominant among all DDR alterations. Unlike truncating alterations that remarkably affect tumor cells by the reduction of gene expression, missense alterations

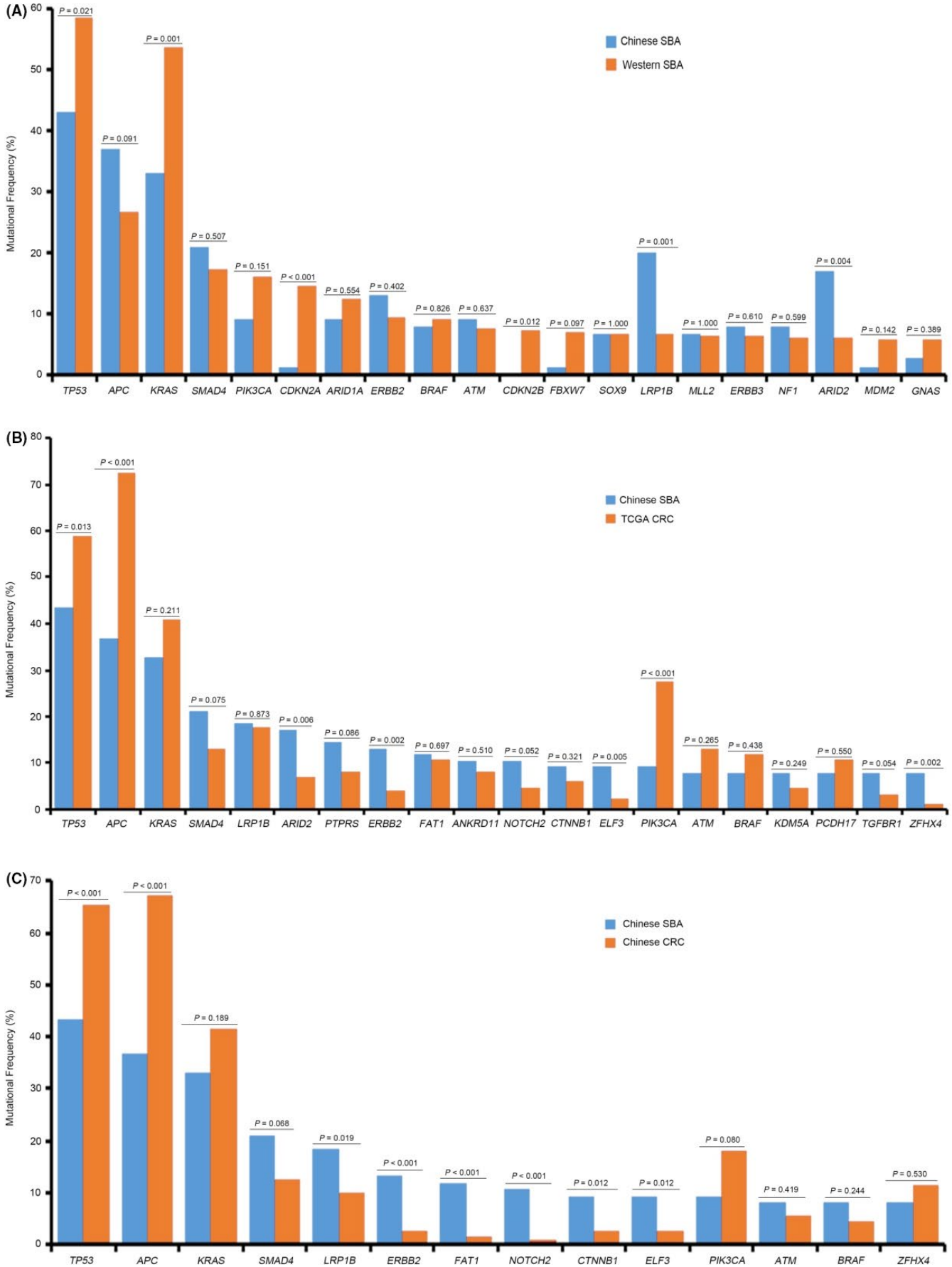
(A)
Mutational Frequency (%)
Chinese SBA
Western SBA
P = 0.021
P = 0.091
P = 0.001
P = 0.507
P = 0.151
P < 0.001
P = 0.554
P = 0.402
P = 0.826
P = 0.637
P = 0.012
P = 0.097
P = 1.000
P = 0.001
P = 1.000
P = 0.610
P = 0.599
P = 0.004
P = 0.142
P = 0.389
TP53 APC KRAS SMAD4 PIK3CA CDKN2A ARID1A ERBB2 BRAF ATM CDKN2B FBXW7 SOX9 LRP1B MLL2 ERBB3 NF1 ARID2 MDM2 GNAS
(B)
Mutational Frequency (%)
Chinese SBA
TCGA CRC
P = 0.013
P < 0.001
P = 0.211
P = 0.075
P = 0.873
P = 0.006
P = 0.086
P = 0.002
P = 0.697
P = 0.510
P = 0.052
P = 0.321
P = 0.005
P < 0.001
P = 0.265
P = 0.438
P = 0.249
P = 0.550
P = 0.054
P = 0.002
TP53 APC KRAS SMAD4 LRP1B ARID2 PTPRS ERBB2 FAT1 ANKRD11 NOTCH2 CTNNB1 ELF3 PIK3CA ATM BRAF KDM5A PCDH17 TGFBR1 ZFHX4
(C)
Mutational Frequency (%)
Chinese SBA
Chinese CRC
P < 0.001
P < 0.001
P = 0.189
P = 0.068
P = 0.019
P < 0.001
P < 0.001
P < 0.001
P = 0.012
P = 0.012
P = 0.080
P = 0.419
P = 0.244
P = 0.530
TP53 APC KRAS SMAD4 LRP1B ERBB2 FAT1 NOTCH2 CTNNB1 ELF3 PIK3CA ATM BRAF ZFHX4

FIGURE 2 Comparison of genomic landscape between Chinese small bowel adenocarcinoma (SBA), Western SBA, and colorectal cancer (CRC) cohorts. A, Comparison of mutational frequencies between Chinese and Western SBA cohorts. B, Comparison of mutational frequencies between Chinese SBA and TCGA CRC cohorts. C, Comparison of mutational frequencies between Chinese SBA and Chinese CRC cohorts

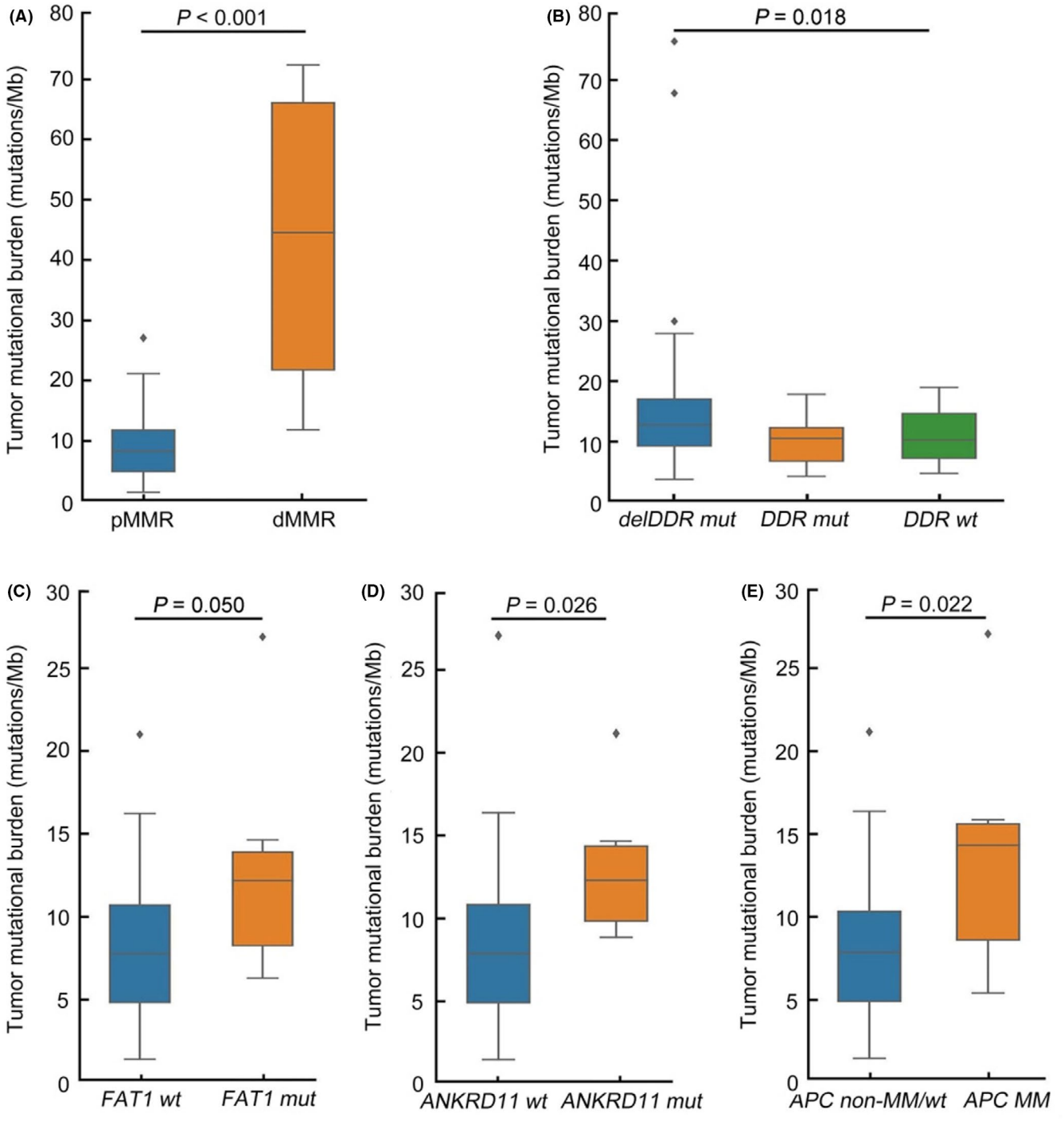

FIGURE 3 Association between TMB, mismatch repair (MMR) status, and certain specific gene mutations. A, Comparison of TMB between mismatch repair–proficient (pMMR) and mismatch repair–deficient (dMMR) patients with small bowel adenocarcinoma (SBA). B, TMB distributions based on DNA damage repair (DDR) mutation status. C-E, Among pMMR patients, comparison of TMB between patients with and without *FAT1*, *ANKRD11*, and *APC* multiple or missense mutations. DDR mut, nondeleterious DDR mutations; DDR wt, nondetectable DDR mutations; delDDR mut, deleterious DDR mutations; MM, multiple or missense mutations; mut, mutations; wt, wild-type

might be benign or deleterious owing to their impacts on protein structures. To avoid the benign alterations that do not influence the protein function, we further divided the DDR alterations into deleterious or nondeleterious groups. All loss-of-function DDR alterations (including frameshift, nonsense, or splice site), and missense alterations defined as pathogenic by the Catalogue of Somatic Mutations in Cancer (COSMIC) or ClinVar database or with a PolyPhen-2 score of ≥0.95 ("probably damaging"), were classified as deleterious.[17] In the study, 24 (31.6%) SBA tumors carried DDR deleterious alterations. We identified a significantly higher level of TMB in the DDR deleterious group than in the DDR nondeleterious/wt group (Figure 3B). Furthermore, among DNA mismatch repair–proficient (pMMR) tumors, *FAT1* and *ANKRD11* alterations were remarkably associated with high TMB (Figure 3C,D). In the present study, different mutation types (missense, nonsense, and INDELs) and multiple mutations (mutation numbers ≥2) of *APC* were observed in some patients (Figure 1A). Further analysis showed a significant association between multiple or missense mutations of *APC* and high TMB in pMMR patients (Figure 3E).

3.4 | Relationship between genomic alterations and survival outcomes

As SBA is generally diagnosed at an advanced stage and little is known about its molecular profile, no studies explore the molecular markers for recurrence in surgically resected patients currently. In this study, recurrence-free survival (RFS) data were collected from 64 cases, and the prognostic significance of the frequently mutated genes was evaluated. Patients with *KRAS* mutations exhibited a worse RFS compared with those of the wt group (hazard ratio [HR] = 2.05, 95% confidence interval [CI] = 0.89-4.69, $P = .044$; Figure 4A). After taking into account age, gender, ECOG performance status, T stage, and clinical stage, *KRAS* mutations remained an independent predictor of RFS (HR = 2.54, 95% CI = 1.14-5.63, $P = .022$; Table 2). Of note, patients with *TP53* mutations tended to have a longer RFS compared with other patients (HR = 0.60, 95% CI = 0.30-1.22, $P = .160$; Figure S7A). In other types of cancer, many studies have demonstrated that *TP53* mutations could be classified as disruptive or nondisruptive, according to their functional effects on the p53 protein, and patients with various mutant categories of *TP53* had a different prognosis.[18,19] Therefore, we further analyzed the association between different types of *TP53* mutations and RFS. Among patients with *TP53* mutations, disruptive *TP53* mutations were correlated with a better RFS (Figure S7B). No statistical difference was observed between patients with nondisruptive *TP53* mutations and patients without *TP53* mutations (Figure S7C). Further analysis showed that patients with disruptive *TP53* mutations exhibited a longer RFS compared with the rest of the patients (Figure S7D). Next, *TP53* and *KRAS* were grouped together in a single category, and its predictive prognostic value was evaluated. All patients were classified into four subgroups: *KRAS* mutations/*TP53* disruptive mutations ($KRAS^{mut}/TP53^{dis}$), *KRAS* mutations/*TP53* wt/nondisruptive mutations ($KRAS^{mut}/TP53^{wt/non\text{-}dis}$), *KRAS* wt/*TP53* disruptive mutations ($KRAS^{wt}/TP53^{dis}$), and *KRAS* wt/*TP53*wt/nondisruptive mutations ($KRAS^{wt}/TP53^{wt/non\text{-}dis}$). Among the four subgroups, $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ exhibited the worst RFS (Figure S8A). Apart from the $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ subgroup, no statistical difference in RFS was identified among the other three subgroups (Figure S8B). Patients with $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ were significantly associated with a poor RFS, as compared with the rest of the patients (HR = 4.07, 95% CI = 1.45-11.44, $P < .001$; Figure 4B). Moreover, following multivariable adjustment, $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ remained an independent negative predictor of RFS (HR = 4.21, 95% CI = 1.94-9.14, $P < .001$; Table 2).

3.5 | Overview of clinically actionable alterations

The profile of clinically actionable mutations was systematically evaluated based on the OncoKB classification system.[20] In total, 118 potential drug-related targets from 57 patients (75%) were identified. Overall, 19 patients (25%) had no drug-sensitive mutations, 19 (25%) had one, and 38 (50%) harbored ≥2 (Figure S9). Alterations in *KRAS*, *ERBB2*, *PIK3CA*, *ATM*, *BRAF*, and *NF1* were the most common targets (Figure 5A). With the OncoKB classification system, we further assigned levels of clinical actionability to targetable alterations in SBA.

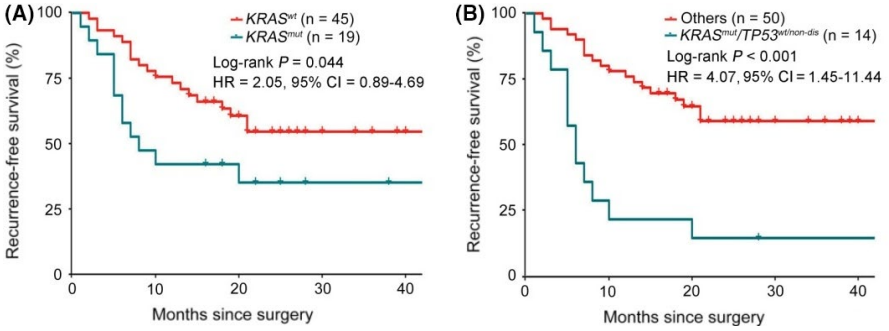

FIGURE 4 Effect of *KRAS* and *TP53* mutation status on recurrence-free survival (RFS). A, Kaplan-Meier estimates of RFS for patients stratified by *KRAS* mutation status. B, Kaplan-Meier estimates of RFS for patients stratified by *KRAS*/*TP53* mutation status. $KRAS^{mut}$, *KRAS* mutations; $KRAS^{mut}/TP53^{wt/non\text{-}dis}$, *KRAS* mutations/*TP53* wild-type/nondisruptive mutations; $KRAS^{wt}$, *KRAS* wild-type; mut, mutation; PS, performance status; TNM, tumor-node-metastasis; wt, wild-type

TABLE 2 Univariate and multivariate analysis of factors associated with recurrence-free survival

		Univariate analysis			Multivariate analysis		
Characteristics	**Parameters**	**HR**	**95% CI**	***P* value**	**HR**	**95% CI**	***P*-value**
Age	>60 vs ≤60	1.52	0.75-3.09	.252			
Gender	Male vs female	1.47	0.72-2.97	.279			
ECOG PS	≥2 vs 0-1	1.16	0.57-2.35	.683			
T stage	T3/T4 vs T1/T2	1.55	0.72-3.32	.296			
Clinical stage	III/IV vs I/II	1.88	0.91-3.86	.071			
KRAS mutations	$KRAS^{mut}$ vs $KRAS^{wt}$	2.05	0.89-4.69	.044	2.54	1.14-5.63	.022
Combination of *KRAS* and *TP53* mutations	$KRAS^{mut}/TP53^{wt/non\text{-}dis}$ vs others	4.07	1.45-11.44	<.001	4.21	1.94-9.14	<.001

Abbreviations: $KRAS^{mut}$, *KRAS* mutations; $KRAS^{mut}/TP53^{wt/non\text{-}dis}$, *KRAS* mutations/*TP53* wild-type/nondisruptive mutations; $KRAS^{wt}$, *KRAS* wild-type; PS, performance status.

OncoKB categorizes different levels of actionability to genetic mutations based on the level of evidence that the alteration is a predictive indicator for drug response, which is of great significance in guiding medication options. As patients with *KRAS* and *NRAS* alterations are ineligible for anti-EGFR therapy, we stratified these alterations into resistant markers. With the approval of PD-1/PD-L1 inhibitors for dMMR solid tumors, dMMR was defined as actionable (level-1). Beyond *KRAS* and *NRAS* mutations, 22 (28.9%) SBA patients harbored highest-level alterations of clinical actionability (level-1 and level-2) (Figure 5B), and *ERBB2*, *BRCA1/2*, and *C-KIT* were the most common targets. Additionally, six patients harboring *BRAF* mutations were identified, whereas only one patient harbored a *BRAF V600E* mutation; the other five patients harbored uncommon *BRAF* mutations with *D594H*, *R362X*, *W531C*, *L597R*, and *V226L* (Figure S10A), which showed some differences with the *BRAF* mutation sites identified in a previous study of a Western population (Figure S10B).[10] A clinically significant *NTRK3-ETV6* rearrangement was identified. Although previous large-cohort studies have investigated the distributions of *NTRK* fusions in various tumors,[21] *NTRK* fusions were never revealed in SBA. *EGFR L858R* alteration was also identified in a patient, which might sensitize the tumor to EGFR tyrosine kinase inhibitors. Moreover, 23.7% of SBA patients had level-3 actionable mutations (Figure 5B), and *PIK3CA* mutations were the most frequently actionable alterations. Pairwise associations between actionable alterations showed co-mutations of *PI3KCA* and *KRAS*, *MTOR* and *ARAF*, *MTOR* and *NF1*, and *AKT1* and *C-KIT*, indicating that genomic alterations in the PI3K/AKT signaling pathway tended to coexist with alterations of other signaling pathways in SBA. Moreover, co-mutations of *MAP2K1* and *FGFR1*, and *MAP2K1* and *FGFR3* were observed, implying that combined genetic alterations within the MAPK signaling pathway might generally occur simultaneously in SBA (Figure 5C).

3.6 | Characterization of microbial community in SBA

High-throughput technology has led to considerable advances in the analysis of the role of bacteria in cancers.[22] However, human SBA microbiome has never been delineated. In this study, bacterial 16s rRNA gene sequencing was conducted on 44 SBA cases. A total of 22 phyla were detected. *Proteobacteia*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Cyanobacteria* were the most common phyla in the study samples. The association between microbiome and clinical characteristics in SBA was investigated. At the phylum level, the relative abundance of *Bacteroidetes* was significantly lower in stage I patients than in stage II-IV patients (Figure S11A). *Proteobacteria* was found to be significantly more abundant in the patients with duodenum adenocarcinoma than in the patients with ileum/jejunum adenocarcinoma (Figure S11B). Moreover, the potential microbiome phenotypes were predicted and compared with BugBase.[23] Among all the phenotypes, the anaerobic phenotype was enriched in stage II-IV patients (Figure S11C), whereas the facultatively anaerobic phenotype was enriched in stage I patients (Figure S11D). In addition, the anaerobic phenotype showed significant differences between duodenum adenocarcinoma and ileum/jejunum adenocarcinoma (Figure S11E). On the basis of relative phyla abundance, all patients were clustered into three subgroups. The most abundant phyla in subgroup I, II, and III were *Proteobacteia*, *Actinobacteria*, and *Firmicutes* and *Bacteroidetes*, respectively (Figure 6A). We further sought to determine if there were differences in the distributions of the subgroups between patients with high risk (RH) and low risk (RL) of recurrence stratified by *TP53* and *KRAS* mutations. The result showed a significant difference in the distributions of the subgroups between RH and RL patients ($P = .044$). RL patients were mainly subgroup I tumors (74.3%) and RH patients were mainly subgroup II/III tumors (66.7%) (Figure 6B).

4 | DISCUSSION

Given the rarity of SBA, genomic and bacterial profiles for this population remain largely unknown. In this study, we examined not only the genomic alterations but also the bacterial community in Chinese patients with SBA. Furthermore, a distinct molecular subtype associated with RFS was revealed. Our study represents the first report to investigate the association of genomic alterations and tumor

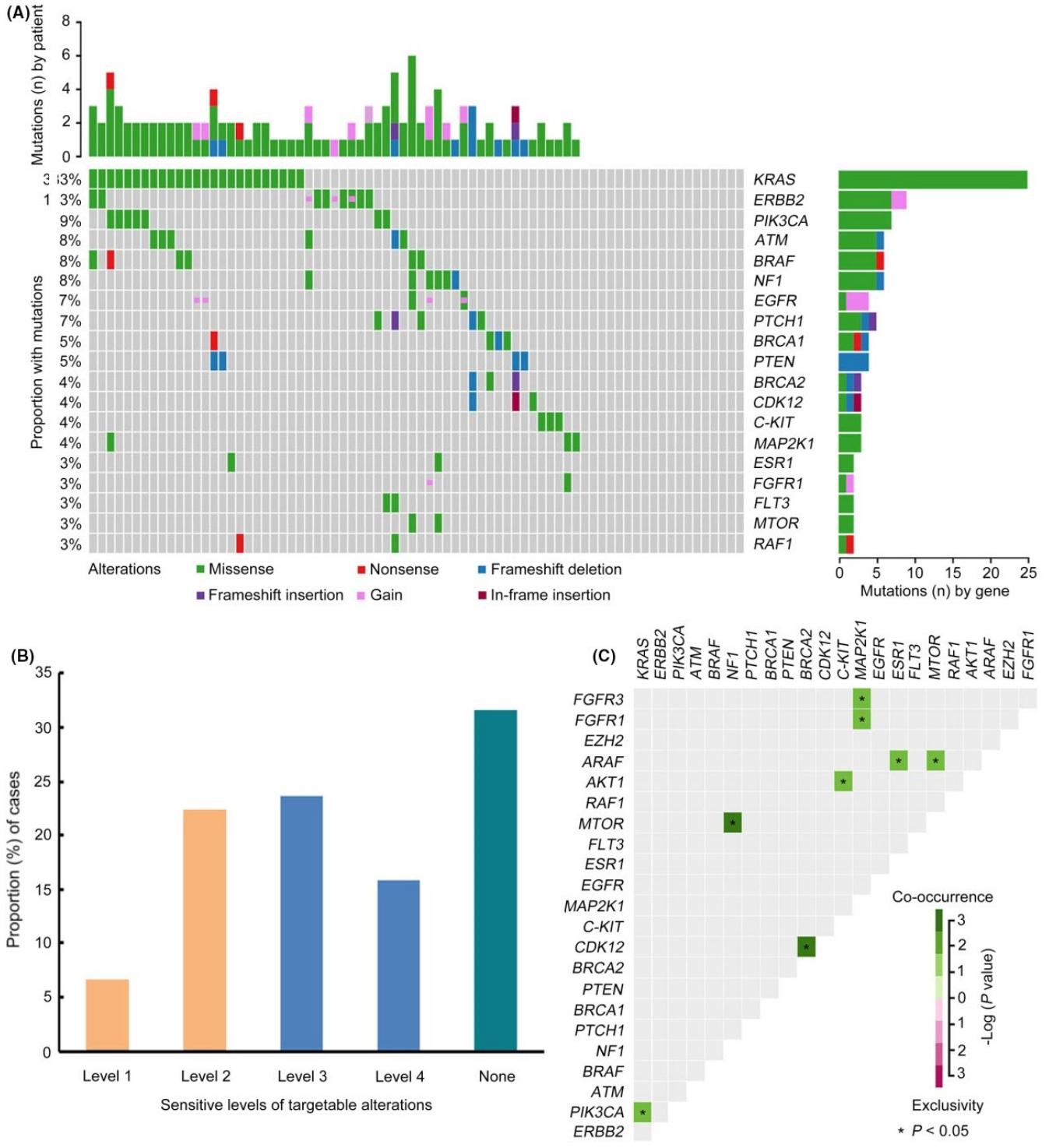

FIGURE 5 Overview of actionable alterations in small bowel adenocarcinoma (SBA). A, Landscape of potentially targetable alterations. B, Distributions of different actionable levels to targetable alterations stratified by the OncoKB classification system. C, Pairwise association plot for actionably mutated genes

microbiome with clinical outcomes in SBA, which will promote the clinical management of SBA.

Herein, significant differences in the genomic landscape were observed among Chinese SBA, Chinese CRC, and TCGA CRC cohorts. Moreover, a different molecular profile was unveiled between Chinese SBA and Western SBA cohorts. These results indicated the specificity of genomic signatures in Chinese patients with SBA. Therefore, it should be taken into consideration when developing new therapeutic strategies targeting Chinese SBA patients. The DDR system is required to maintain the genome integrity, and DDR deficiency is generally caused by mutations in checkpoints or DDR protein.[24] In this study, we found 55.3% of SBA patients harbored

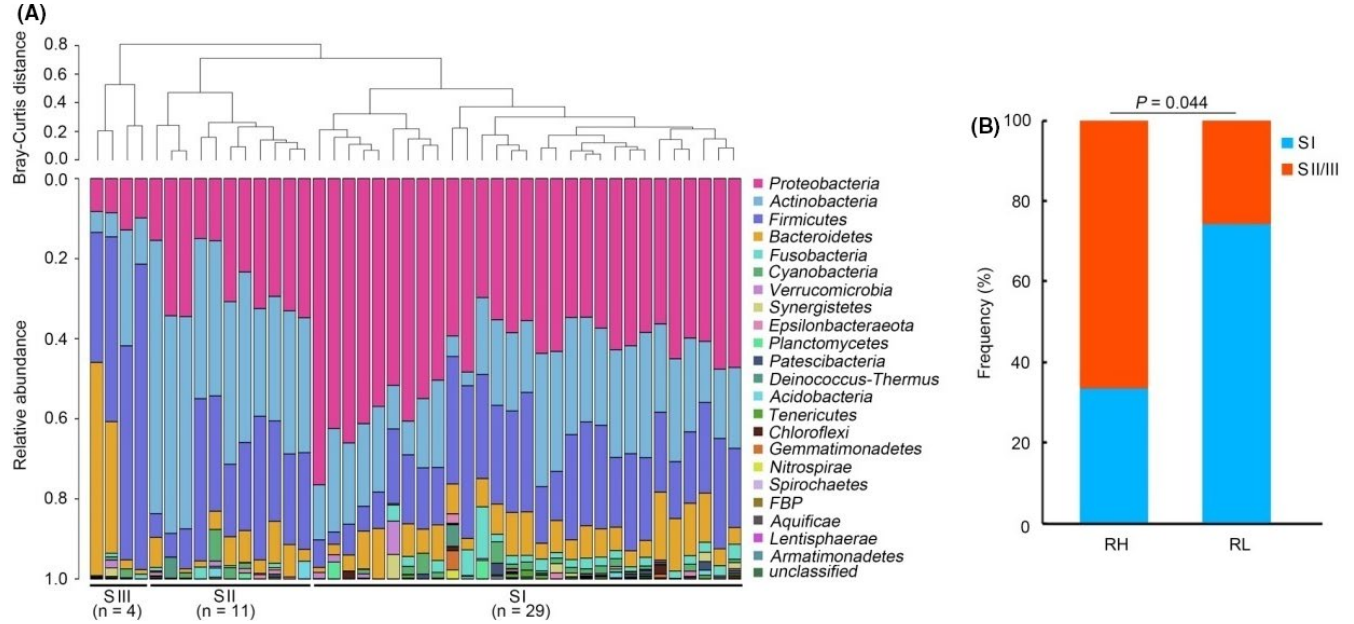

FIGURE 6 Tumor microbiome communities in small bowel adenocarcinoma (SBA). A, Bar plots of the phyla taxonomic levels in SBA and the subgroups categorized by relative abundance of bacterial phyla. B, Association between the distributions of the subgroups and the risk of recurrence stratified by *TP53* and *KRAS* mutations. RH, high risk of recurrence; RL, low risk of recurrence; SI, subgroup I; SII, subgroup II; SIII, subgroup III

genetic mutations of the DDR pathway, indicating a crucial role of DDR deficiency during the development of SBA. Among the mutational signatures, MMRD signature was commonly seen in CRC. However, HRD signature was rare in primary CRC patients.[25] Although there was a similarity between the ancestry mutations of SBA and CRC, the SBA still displayed a diverse mutational pattern. Therefore, it is urgent to comprehensively explore and understand the genomic spectrum of SBA, and future treatment strategies for SBA should be based on its own molecular features rather than on those of CRC.

In our study, 5.3% (4/76) of Chinese SBA patients exhibited dMMR status. Recently, a study investigated the relationship between dMMR and Lynch syndrome in Western SBA patients and revealed that 26% of SBAs showed dMMR status and Lynch syndrome prevalence was 38.5% among dMMR SBAs.[26] However, none of dMMR SBAs was Lynch syndrome in our study cohorts. Overall, the prevalence of dMMR status and Lynch syndrome in our SBA cohorts was lower than that in Western SBA cohorts of a recent study.[26] One explanation for these findings is the population differences. A previous study reported that microsatellite instability-high (MSI-H) prevalence was 7.6% in a large Western SBA cohort,[13] which was similar to that of our cohorts. Therefore, although there was no difference between the two populations in different studies,[13,26] the dMMR prevalence was still remarkably different, not to mention different population-based SBA cohorts. Additionally, the relatively small number of dMMR SBAs in our cohorts could influence the Lynch syndrome prevalence estimates. Therefore, the association between dMMR and Lynch syndrome in a large Chinese SBA cohort needs to be further investigated in the future.

It has been identified that dMMR tumors have a high response to immune checkpoint inhibitor in various cancer types, including SBA.[27] Moreover, a recent clinical trial of pembrolizumab in an unselected advanced SBA population was conducted.[28] In this study, candidate predictive markers of response, including dMMR status and high TMB were identified, whereas no association was found between PD-L1 expression and response rate to pembrolizumab.[28] For our SBA cohorts, only a small number of tumors exhibited dMMR status. Therefore, a full understanding of TMB and its correlation with other factors is of great significance to guide future immunotherapy in SBA. Herein, dMMR tumors harbored a high TMB, which was similar to other types of cancer.[29] DNA repair deficiency has become an emerging predictive factor of response to immunotherapy, and alterations in DDR genes are associated with higher genomic instability and elevated TMB, which might increase immunogenicity through enhancing tumor neoantigen load.[16] We found 31.6% of SBA patients harbored DDR deleterious mutations and firstly revealed it was significantly associated with high TMB. Patients with DDR deleterious alterations were recently shown to benefit from PD-1/PD-L1 blockade in advanced urothelial and non–small cell lung cancer.[17,30] Therefore, future studies are required to determine the relationship of DDR deleterious alterations with clinical outcome to immunotherapy in SBA. In addition to dMMR tumors, we observed certain pMMR tumors also harbored a high TMB. A recent large-scale study reported that microsatellite-stable tumors with a high TMB could benefit from treatment with PD-1/PD-L1 blockade, further confirming that TMB was associated with the efficacy of immunotherapy.[31] This study revealed that pMMR tumors with *FAT1*, *ANKRD11*, and *APC* multiple/missense mutations harbored a higher overall TMB.

Although more research will be indispensable to validate these findings, our results provided novel biomarkers that might help to guide future design of personalized immunotherapy trials in SBA.

Due to the low incidence and generally advanced stage at presentation of SBA, there is a striking lack of available studies deciphering molecular characterization and prognostic indicators simultaneously in SBA, and no reports assessed risk stratification biomarkers for recurrence in surgically resected patients with SBA currently. This study investigated in length the associations between specific gene mutations and RFS. *TP53* was the most commonly mutated gene in SBA. Patients with disruptive *TP53* mutations exhibited a longer RFS than other patients. In SBA, a previous large-cohort study indicated that younger age was significantly associated with a better prognosis.[32] In our study cohorts, patients with disruptive *TP53* mutations were younger than patients with nondisruptive *TP53* mutations and without *TP53* mutations (median age: 56.5 years vs 64.0 years). Further analysis showed that patients with disruptive *TP53* mutations exhibited a higher proportion of good ECOG performance status (ECOG PS of 0-1), as compared with the rest of the patients (65.0% vs 47.7%). This could explain that disruptive *TP53* mutations were associated with a better prognosis to some extent. Moreover, experimental evidence demonstrates that some nondisruptive *TP53* mutations induce "gain of function" (GOF) activities, rather than resulting in simple loss of function of wt p53. These GOF activities, which can be shown through direct inactivation or transcriptional regulation of p63/p73, are dominant over the *TP53*-wt allele and cause elevated cell growth rate, metastasis, invasiveness, and tumorigenicity.[18,33,34] However, disruptive *TP53* mutations are less likely to acquire GOF activities. These results could further explain the better prognosis observed in patients harboring disruptive *TP53* mutations. Further analysis demonstrated that *KRAS* mutations in SBA were an independent predictor of a poor RFS. Therefore, the prognostic significance of combined *KRAS* and *TP53* mutation status was evaluated. Based on the results, $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ was independently associated with a worse prognosis (HR = 4.21, 95% CI = 1.94-9.14), which markedly improved the RFS risk estimates, as compared with *KRAS* mutations (HR = 2.54, 95% CI = 1.14-5.63). To the best of our knowledge, this study is the first report to identify a prognostic indicator for recurrence in SBA patients undergoing surgical resection based on the combined *KRAS* and *TP53* mutation status.

Despite significant advances in understanding the pathogenesis and therapeutic strategies of human malignancies, there is no standard therapy, and clinical trials investigating novel treatment strategies are limited in SBA. Our data showed 75% of SBA patients carried potentially targetable alterations. To further comprehensively estimate the clinical application of genomic profiling to guide treatment decisions in SBA, we classified all the targetable alterations into different levels of clinical actionability using OncoKB system. Altogether, 28.9% of SBA patients harbored highest-level alterations of clinical actionability, of which *ERBB2* mutations were the most commonly highest-level actionable alterations in SBA. A recent work in Western populations has revealed that *ERBB2* alterations could be regarded as novel personalized treatment options in SBA.[9] Therefore, further studies and clinical trials are necessary to prove the efficacy of this targeted therapy in SBA. *BRCA1* and *BRCA2* mutations also accounted for a high percentage of highest-level actionable alterations, which was consistent with the specific mutational signature of HRD identified in the study. Currently, many clinical trials have demonstrated that various human malignancies with *BRCA* mutations, respond well to PARP inhibitors.[35] A recent study reported that *BRCA2* alterations were potential targets in SBA.[10] Our results once again confirmed the finding and further uncovered that *BRCA1* mutations were novel important targetable candidates in SBA. Moreover, *C-KIT* mutations were critical potential targets identified in Chinese SBA cohorts, which generally occurred in gastrointestinal stromal tumors and melanomas.[36] Many drugs targeting *C-KIT* mutations are being developed. As prior work tended to have small sets of samples and focused on a limited number of genes, *C-KIT* mutations have never been revealed in SBA. Notably, *BRAF* mutations in Chinese cohorts were different not only from those in CRC but also from those in Western SBA cohorts.[10] Therefore, pan-RAF or MEK inhibitors might be potential targeted drugs for *BRAF* mutations in SBA. Furthermore, level-3 alterations such as *PIK3CA*, *PTEN*, *ATM*, *EGFR*, and *MAP2K1* also play potential roles in informing individual treatment decisions in SBA. Thus, it is essential to perform multigene testing in SBA, which is of great importance to guide personalized therapy in this population.

Numerous studies indicate that some microbiota are associated with cancer. For instance, *Chlamydia trachomatis* and *human papillomavirus* are known to be associated with cervical cancer.[37,38] However, the microbial community in human SBA has never been reported. Our study revealed that *Proteobacteia*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria*, and *Cyanobacteria* were the most common phyla in SBA, and the tumors could be classified into three subgroups according to the phyla abundance. Additionally, the distributions of the subgroups were associated with the risk of recurrence stratified by *TP53* and *KRAS* mutations, which further indicated the prognostic significance of the distinct molecular subtype of $KRAS^{mut}/TP53^{wt/non\text{-}dis}$ in SBA.

In conclusion, the present study systematically depicted the genomic and bacterial profiles of Chinese patients with SBA, which provided an enhanced roadmap for understanding this rare cancer. For the first time, we conducted an in-depth analysis on assessing the molecular markers for recurrence risk stratification in SBA. Furthermore, TMB assessment and the classifications of targetable mutations based on different levels of clinical actionability represent a critical step forward for promoting the development of treatment strategies and clinical management of patients with SBA.

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DISCLOSURE

Huina Wang, Feng Lou, and Shanbo Cao are employees of Acornmed Biotechnology Co., Ltd. The other authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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