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RNF43 frameshift mutations contribute to tumourigenesis in right-sided colon cancer

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ABSTRACT

The truncating mutations of RNF43 frequently occur in CRC, we aimed to clarify the relationship between RNF43 frameshift mutations and MS status, BRAF V600E mutation, distant metastasis, TNM stage and location of CRC. RNF43 frameshift mutations R117.fs and G659.fs and BRAF V600E mutation were detected in 392 sporadic CRC samples from our tissue bank. We integrated our original study with the TCGA database and five published datasets to analyse the relationship between RNF43 frameshift mutation and tumour location, distant metastasis, TNM stage and BRAF V600E mutation in 2396 CRC samples when controlling for MS status. RNF43 frameshift mutation was correlated with MSI-H (OR = 122.27) [31.82, 469.92], BRAF V600E mutation (OR = 7.92 [3.45, 18.18]), distant metastasis (OR = 0.30 [0.17, 0.53]), advanced TNM stage (OR = 0.34)[0.23, 0.51], and right colon site (OR = 8.32 [2.98, 23.22]). After controlling for the effect of MS status, there was no correlation of RNF43 frameshift mutation with distant metastasis (OR = 1.57 [0.75, 3.28]) and advanced TNM stages (OR = 0.98 [0.58, 1.67]), but RNF43 frameshift mutations still occur more frequently in right colon (OR = 2.58 [1.49, 4.47]) and with BRAF V600E mutation (OR = 1.94 [1.22, 3.10]). RNF43 frameshift mutations were related to distant metastasis and TNM-stage in an MS status-dependent manner, but they contributed to tumourigenesis in right-sided colon cancer independent of MS status.

1. Introduction

RNF43 is a single transmembrane ring-type E3 ubiquitin ligase located at chromosome 17q22 and consisting of 783 amino acids in humans. RNF43 can selectively ubiquitinate frizzled receptors through targeting surface-expressed frizzled receptors to lysosomes [1] and sequester TCF4 to the nuclear membrane to inhibit the downstream signal of mutated β-catenin [2], thus negatively regulating the Wnt signal pathway. Additionally, RNF43 was found to be regulated by the Wnt pathway through negative feedback [3]. RNF43 has been identified as a tumour suppressor in multiple types of cancer, including ovarian cancer [4], gastric cancer [5,6], pancreatic cancer [7], endometrial cancer [8] and colorectal cancer [5,8,9]. Moreover, the mutations of RNF43 have been frequently detected in cholangiocarcinoma [10], ovarian cancer [11], gastric cancer [8], endometrial cancer and colorectal cancer [8] and have been considered to be driver mutations of carcinogenesis.

CRC has the third highest incidence and mortality of all cancers worldwide and in the USA [12]. It is well-established that the ectopic activation of the Wnt signal pathway plays an important role in the early stage of the colorectal adenoma-carcinoma sequence [13,14]. More than 90% of colorectal cancers bear Wnt signalling component mutations [14]. The mutation rate of RNF43 has been reported to be up to 17.6% (49/222) in CRC [8]. Among these mutations, the truncating mutations of RNF43 occurred in 79.7% of MSI-H colorectal cancers, and more than half of RNF43 mutations in MSI-H colorectal cancers were the frameshift mutations p.Gly659fs and p.Arg117fs [8]. However, RNF43 mutations rarely occur simultaneously with APC mutations and RSPO3-PTPRK fusion, suggesting that RNF43 inactivation may be one of the key driving events for colorectal carcinogenesis [8,15].

Recently, RNF43 mutations were identified to be associated with

Abbreviations: CRC, colorectal cancer; MSI-H, microsatellites high instability; MSI-L, microsatellites low instability; MSS, microsatellites stable; MS, microsatellites; OR, odds ratio

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BRAF mutation in both the sporadic and familial serrated polyposis syndrome, which could provide a genetic diagnosis protocol and raise therapeutic opportunities for serrated neoplasia. However, it remains controversial whether serrated polyposis is even associated with RNF43 mutations [16], and a large multinational study provided no evidence for RNF43 mutations in patients with serrated polyposis syndrome [17]. Although serrated polyps are considered precancerous lesions of colorectal cancer, the clinical significance of RNF43 mutations still requires further study and exploration in colorectal cancer. In this study, we systematically analysed the hotspot frameshift mutations (RefSeq NP_001292473 p.Gly659fs and p.Arg117fs) of RNF43 in 2396 colorectal cancer samples from our tissue bank, the TCGA database, DFCI 2016, the CRC MSK 2018 dataset, and the data extracted from three publications (Net Genet 2014, J Pathol 2016 and Gut 2017) [18–20], which uncovered the relationship between RNF43 mutations and tumour site, distant metastasis, TNM stage, microsatellite status and BRAF V600E mutation.

2. Methods

2.1. Original study

Formaldehyde-fixed-paraffin embedded (FFPE) tissues originating from 392 sporadic colorectal cancer tumours were obtained from study patients, who were enrolled from 2007 to 2008 at Zhejiang University Affiliated First Hospital. The genome DNA was isolated using a QIAGEN FFPE Tissue Genome Isolation Kit.

The microsatellite status was detected by fluorescent multiplex PCR-capillary electrophoresis (FM-CE). The five loci BAT25, BAT26, D5S346, D2S123 and D12S250 were detected. If a sample had 2 or more loci demonstrating instability, it was defined as MSI-H, and if the sample had one locus displaying instability, then it was defined as MSI-L; samples with no loci exhibiting instability were defined as MSS.

The RNF43 frameshift mutation Arg117fs (RefSeq NP_001292473 p.Arg117fs) in exon3, Gly659fs (RefSeq NP_001292473 p.Gly659fs) in exon 9 and BRAF V600E (RefSeq NP_001341538 p.V600E) were detected using PCR and Sanger sequencing with specific primers for p.Arg117fs (5'-TCCCACCCGCTGTACCTGTGC-3' and 5'-TGTCAAAGAG GACAGCACTGG-3'), p.Gly659fs (5'-GCAAAAATCCAGCCTCTCTGC-3' and 5'-GGGGACCAAGGATATGCCAC-3') and BRAF RefSeq NP_001341538 V600E (5'-CCTACAGGAAGCAAGTAGTAA-3' and 5'-ATGTACTGGTCCCTCATTG-3'). PCR was performed in 12.5 μL Promega GoTaq colourless master mix, 9.5 μL ddH_2O, 1 μL 10 μM forward primer, 1 μL 10 μM reverse primer and 1 μL 200 ng/μl DNA. The reaction mixture was denatured for five minutes at 95℃ and then incubated for 40 cycles (denaturing for 30 s at 95℃, annealing for 40 s at 54℃, and extension for 30 s at 72℃). A final extension was continued for five minutes at 72℃. The protocals (201656) were approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

2.2. Public datasets

There are 8 datasets in total providing data on colorectal carcinoma in cBioPortal. After removing the duplicated datasets and the datasets that contains less than 5 RNF43 frameshift mutations, 3 datasets were used to further analyse the correlation between RNF43 frameshift mutations and clinical characteristic. Among them, the TCGA colorectal cancer mutation data (version 20160128) and clinical data (version 20160128) were downloaded from Firehose. The remaining two datasets DFCI_2016 and MSK_2018 possessing colorectal cancer mutation and clinical data were downloaded from BioPortal.

2.3. Published papers

The following terms were used to search relevant studies in PubMed on Apr 8 2018: “Search item: RNF43 AND ([colorectal cancer] OR [colon cancer] OR [rectal cancer] OR [colorectal adenocarcinoma] OR [colon adenocarcinoma] OR [rectal adenocarcinoma])”. A total of 60 relevant articles were retrieved. By reviewing the title and abstract, we included papers that [1] included RNF43 mutation in colorectal cancer tumour patient samples and [2] were not duplicated with other datasets of cBioPortal. Then, the full text and supplementary data from 7 papers were accessed for further review. The datasets of papers [1] that exhibited RNF43 hotspot R117.fs and G659.fs mutation number less than 5 or [2] for which individual clinical pathological (e.g., tumour site, distant metastasis location, TNM stage, microsatellite status) or mutation data were not available were excluded. As the result, datasets from 3 published papers, including Net Genet 2014, J Pathol 2016 and Gut 2017, were integrated into this study [18–20].

2.4. Statistical analysis

The OR (odds ratio) was calculated for the 2×2 table, and the Fisher exact test was used to perform statistical analysis. We combined the OR using the fixed model unless the heterogeneity Q-test $P < 0.10$, in which case the random-effect model was applied. A forest plot was used to show the pooled result. We used R 3.14 for statistical analysis and the package 'meta' for meta-analysis.

3. Results

3.1. Demographic information of enrolled patients

As shown in Table 1, this study included seven datasets, our local sample (n = 392), the TCGA colorectal cancer dataset (n = 489), the DFCI_2016 colorectal dataset (n = 619), the MSK_2018 dataset (n = 594), NG_2014 (n = 185), JP_2016 (n = 70) and GUT_2017 (n = 47). Patients from TCGA, DFCI_2016 and GUT_2017 were significantly older than the patients in the remaining four datasets. The most common site at which CRC tumours arose was the rectum for the local sample, left colon for TCGA MSK_2018 and JP_2016, and right colon for DFCI_2016 and GUT_2017. The distant metastasis patient ratio (59%) and advanced degree ratio (84%) were higher in MSK_2018. The MSI-H CRC patient rate was higher in the local sample compared with TCGA and DFCI_2016, besides the samples included in GUT_2017 were all MSI-H (100%). The BRAF-V600E mutation rate was higher in DFCI_2016 and JP_2016.

3.2. Association of the RNF43 frameshift mutations with the clinicopathologic characteristics of CRC

RNF43 frameshift mutations were detected in 39 (9.9%) of 392 patients in the local sample, 32 (6.5%) of 489 patients in TCGA, 32 (5.2%) of 619 patients in DFCI_2016, 30 (5.1%) of 594 in MSK_2018, 21 (11.4%) of 185 patients in NG_2014 and 1 (1.4%) of 70 patients in JP_2016. The dataset from GUT_2017 was excluded because the dataset comprised only MSI-H samples. As shown in supplementary table S1, RNF43 frameshift mutations were correlated with tumour site in the local sample, TCGA, DFCI_2016, MSK_2018 and NG_2014 datasets, with distant metastasis in the local sample and MSK_2018 datasets, with TNM stage in the TCGA, MSK_2018 and NG_2014 datasets, with microsatellite status in the local sample, TCGA, DFCI_2016, MSK_2018 and NG_2014 datasets, and with BRAF V600E mutation status in the TCGA, DFCI_2016, MSK_2018 and NG_2014 datasets.

To further confirm the correlation between RNF43 frameshift mutations and these pathological parameters, we performed a systemic meta-analysis for these datasets. The results showed that the RNF43 frameshift mutation was positively related to MSI-H (OR = 122.27, 95% CI = 31.82–469.92, Fig. 1A), BRAF V600E mutation (OR = 7.92, 95% CI = 3.45–18.18, Fig. 1B) and right colon (OR = 8.32, 95% CI = 2.98–23.22, Fig. 1C). However, RNF43 frameshift mutation was

Table 1
Demographic information of enrolled patients.

Characteristics		Local sample N = 392	TCGA N = 489	DFCI_2016 N = 619	MSK 2018 N = 594	NG_2014 N = 185	JP_2016 N = 70	GUT_2017 N = 47
Age								
	≤ 60-No. (%)	179(45.9)	134(27.4)	81(13.1)	382(64.3)	21(11.4)	12(17.1)	21(44.7)
	> 60-No. (%)	211(54.1)	355(72.6)	536(86.9)	213(35.9)	164(88.6)	58(82.9)	26(55.3)
	Missing-No.	2	0	2	0	0	0	0
Gender-No. (%)								
	Male	215(55.3)	225(46.0)	380(61.4)	273(46.0)	75(40.5)	38(54.3)	24(51.1)
	Female	174(44.7)	264(54.0)	239(38.6)	322(54.2)	110(59.5)	32(45.7)	23(48.9)
	Missing-No.	3	0	0	0	0	0	0
Site-No. (%)								
	Right Colon	101(29.5)	175(36.6)	315(51.0)	186(31.5)	104(56.2)	7(10)	32(68.1)
	Left Colon	72(21.1)	216(45.2)	166(26.8)	265(44.9)	52(28.1)	37(52.9)	15(31.9)
	Rectum	169(49.4)	87(18.2)	137(22.2)	140(23.7)	29(15.7)	26(37.1)	/
	Missing-No.	50	11	1	4	0	0	0
Distance metastasis-No. (%)								
	Negative	279(87.2)	377(78.1)	498(88.5)	244(41.1)	144(85.2)	/	/
	Positive	41(12.8)	106(21.9)	65(11.5)	351(59.1)	25(14.8)	/	/
	Missing-No.	72	6	56	0	16	/	/
TNM-No. (%)								
	I/II	204(58.0)	273(57.6)	339(60.2)	97(16.3)	91(55.5)	/	/
	III/IV	148(42.0)	201(42.4)	224(39.8)	498(83.8)	73(44.5)	/	/
	Missing-No.	40	15	56	0	21	/	/
Microsattelite status-No. (%)								
	MSI-H	68(23.5)	71(14.5)	91(17.2)	84(14.1)	31(18.5)	/	47(100)
	MSI-L	52(18.0)	77(15.8)	/		2(1.2)	/	0(0)
	MSS	169(58.5)	340(69.7)	438(82.8)	511(86.0)	135(80.4)	/	0(0)
	Missing-No.	103	1	90	0	17	/	0
BRAF-V600E. (%)								
	Wild type	355(91.0)	441(90.2)	508(82.1)	542(91.2)	144(77.8)	29(41.4)	35(74.5)
	Mutant	36(9.0)	48(9.8)	111(17.9)	53(8.9)	41(22.2)	41(58.6)	12(25.5)
	Missing-No.	1	0(0)	0(0)	0	0	0	0

inversely related to distant metastasis (OR = 0.30, 95% CI = 0.17–0.53, Fig. 1D) and advanced TNM stage (OR = 0.34, 95% CI = 0.23–0.51, Fig. 1E).

3.3. The relationship between RNF43 frameshift mutations and clinicopathologic characteristics when controlling for microsatellite instability

As we know, the MSI-H CRCs predominantly occurred in the right colon, which were correlated with BRAF V600E mutation, less distant metastasis and low TNM-stage [21]. We also verified these results in our study. As shown in Fig. 2, MSI-H is positively related to right colon (OR = 6.28, 95% CI = 3.27–12.09, Fig. 2A) and BRAF V600E mutation (OR = 9.1, 95% CI = 3.32–24.99, Fig. 2B) and negatively correlated with distant metastasis (OR = 0.16, 95% CI = 0.07–0.36, Fig. 2C) and advanced stage (OR = 0.22, 95% CI = 0.14–0.35, Fig. 2D). Interestingly, the MSI-H status contributed to RNF43 frameshift mutation in colorectal cancer. In this study, the correlation between RNF43 frameshift mutation and the MSI-H phenotype was dramatically higher (OR = 122.27) than the effect of other study variables. Moreover, the influence of RNF43 frameshift mutations on these pathological parameters was consistent with MSI-H status. However, it remains unclear whether MSI-H status mediated the correlation between RNF43 frameshift mutation and these clinicopathological characters. Therefore, we only included MSI-H patients and controlled for the effect of MS status to perform a meta-analysis. The results showed that there was no statistical significance for the correlation between RNF43 frameshift mutation and distant metastasis (OR = 1.57, 95% CI = 0.75–3.28, Fig. 3A) or advanced TNM stages (OR = 0.98, 95% CI = 0.58–1.67, Fig. 3B). However, after adjustment for the effect of MSI-H, the RNF43 frameshift mutations continued to occur more frequently in the right colon (OR = 2.58, 95% CI = 1.49–4.47, Fig. 3C) and were significantly related to BRAF V600E mutation (OR = 1.94, 95% CI = 1.22–3.10, Fig. 3D).

To avoid false positives caused by one dominant dataset interfering with the final result, we performed sensitivity analyses by excluding each dataset in turn. The pooled results showed that distant metastasis and advanced TNM stages were not related to RNF43 frameshift mutations; however, tumour site still remained strong correlated with RNF43 frameshift mutations when removing any one of four datasets (Table 2). Unexpectedly, there was no relationship between BRAF V600E mutation and RNF43 frameshift mutations when removing the TCGA dataset (Table 2). Because excluding DFCI_2016 made I^2 decline to 0, we interpreted this as a strong heterogenicity existing for the BRAF V600E mutation between DFCI_2016 and the other datasets. Together with these results, RNF43 p.Arg117fs and p.Gly659fs mutations were strongly related to right-sided colon cancer independent of MS status.

4. Discussion

RNF43 is an important negative regulator of the Wnt signal pathway and part of the WNT negative feedback loop. Impaired RNF43 function may cause an unregulated Wnt cascade [1]. The predominance of RNF43 mutations in oncogenesis has been well-reported. The RNF43 mutation was recurrent in several tumours, including gastric cancer, endometrial cancer, pancreatic cancer and colorectal cancer. It has been proposed that RNF43 mutations were under positive selection and were mutually exclusive with the APC mutations and PTPRK–RSPO3 fusion in CRC patients [8,15]. The RNF43 mutation of hotspots p.Gly659fs and p.Arg117fs represented more than half of the RNF43 mutation frequency in CRC [8]. The RNF43 hotspot mutations in CRC clinical tumor samples displayed relatively less heterogeneity than other mutations, which suggests that they are an early event in the process of oncogenesis or tumour progression [5].

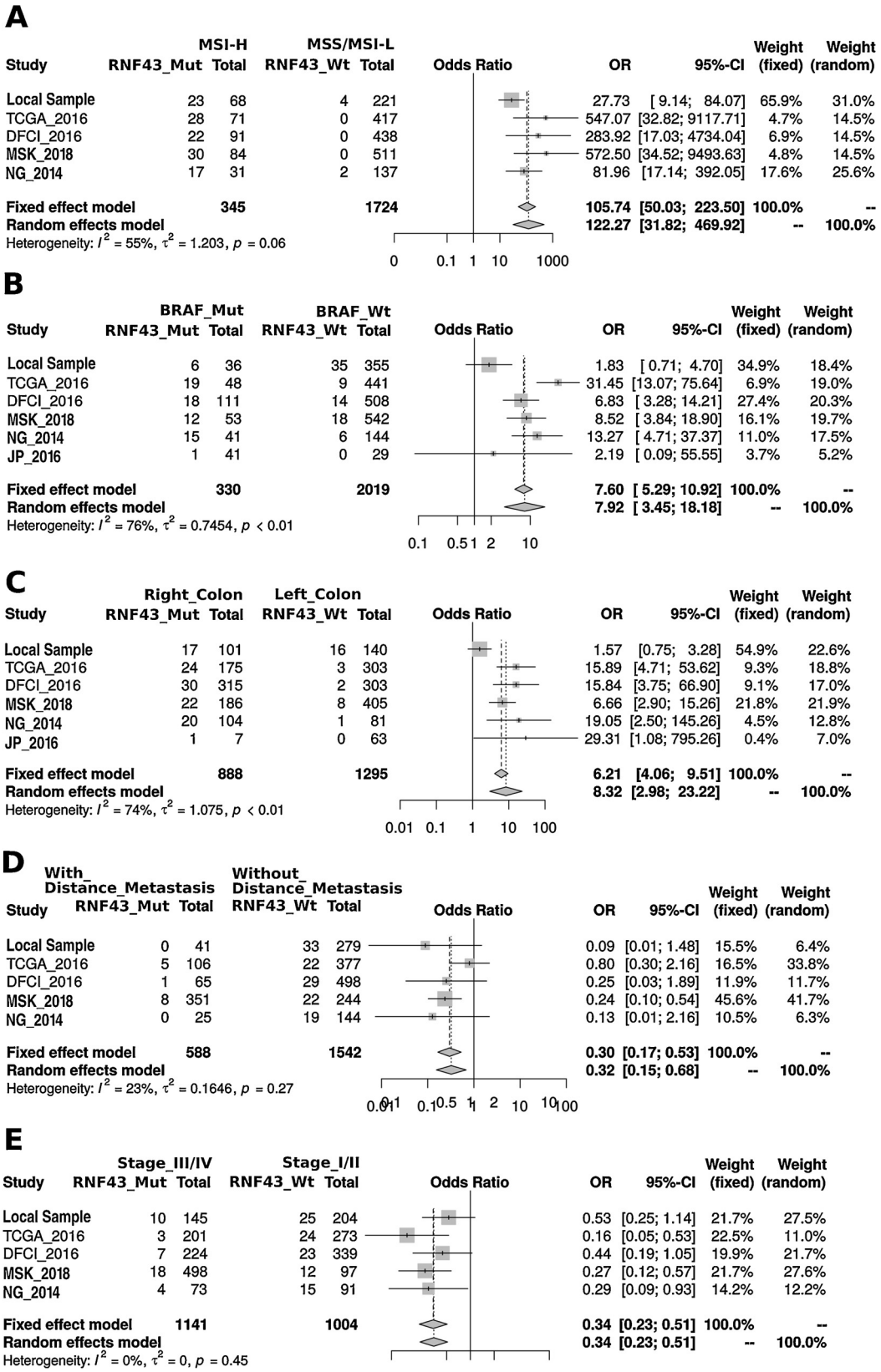

Fig. 1. The relationship between RNF43 frameshift mutations and CRC pathological parameters. Forest plots of the association between RNF43 frameshift mutations and MS status (A), BRAF V600E mutation (B), tumour location (C), distant metastasis (D) and TNM stage (E).

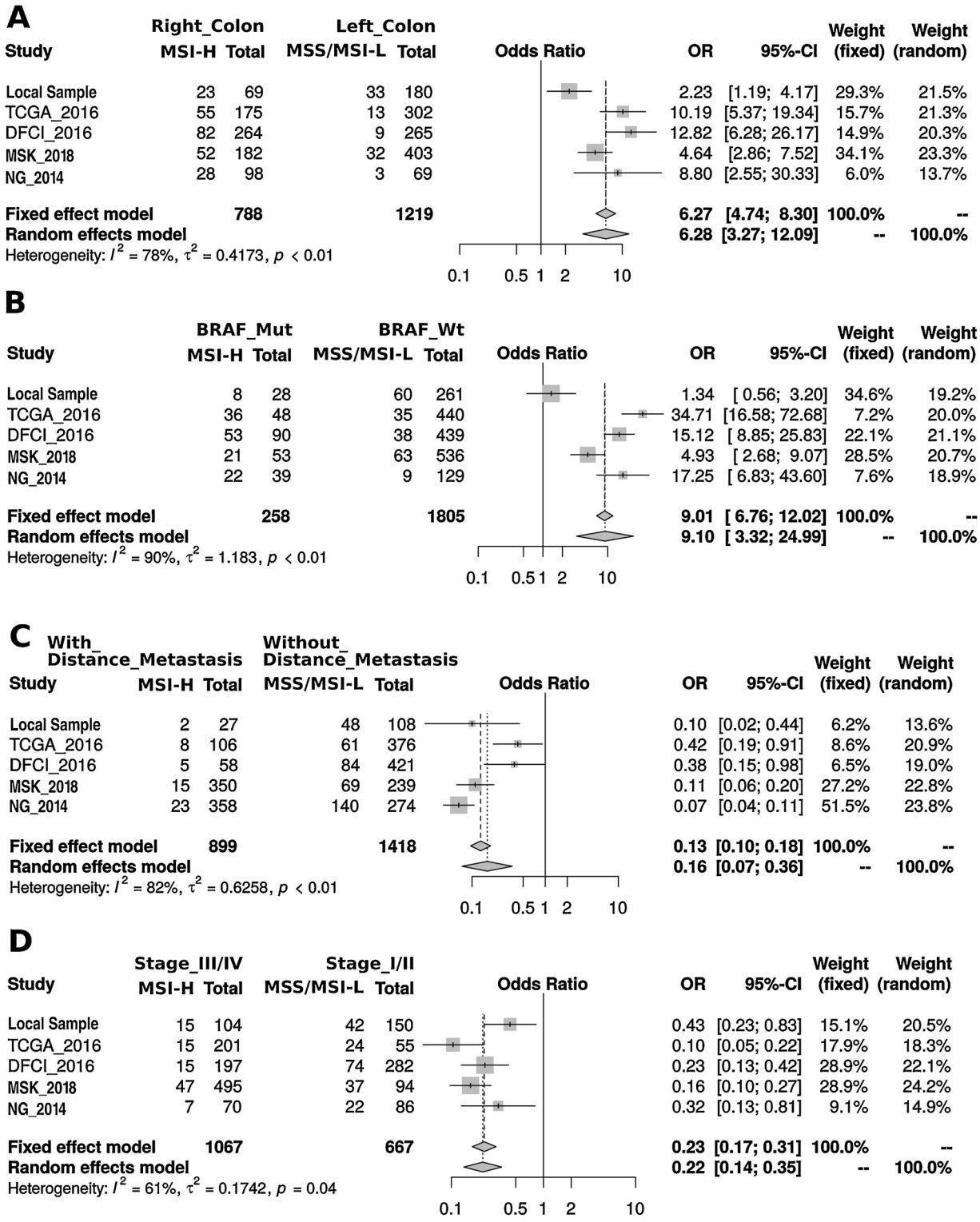

Fig. 2. The relationship between microsatellite status and CRC pathological parameters. Forest plots of the association between MS status and tumour location (A), BRAF V600E mutation (B), distant metastasis (C), and advanced TNM stage (D).

In this study, we explored the relationship between the RNF43 frameshift hotspot mutations (p.Gly659fs and p.Arg117fs) and clinicopathologic variables in CRC. By pooling the mutation data and clinical data from our local sample and the TCGA, DHCI_2016, MSK_2018, NG_2014, JP_2016 and GUT_2017 datasets, we found that the RNF43 frameshift mutations were correlated with MSI-H, BRAF V600E mutation, less distant metastasis, early-stage TNM and a right colon location of the primary tumour. A previous study has observed that the RNF43 frameshift hotspot site mutations are more frequent in MSI-H colorectal cancer [8], and the two hotspot sites are homopolymeric tracts of seven and six C-G pairs, respectively. Moreover, MSI-H colorectal cancer tumours are correlated more frequently with BRAF V600E mutation and favour the right colon for primary tumour location, as well as less distant metastasis and early TNM stage [22–24].

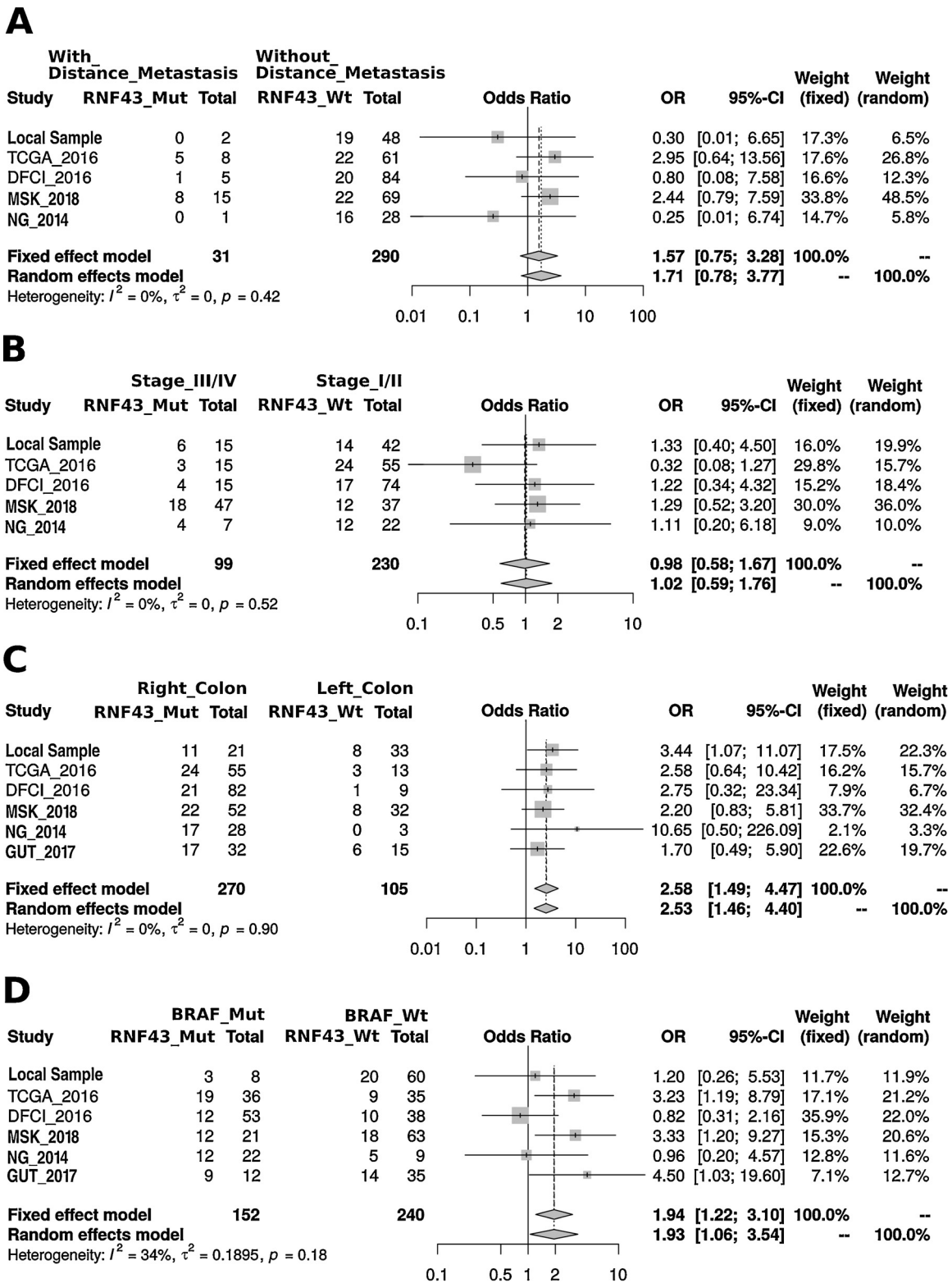

Fig. 3. The relationship between RNF43 frameshift mutations and clinicopathologic characters with microsatellite status controlled. Forest plots of the association between RNF43 frameshift mutations and distant metastasis (A), advanced TNM stage (B), tumour location (C), and BRAF V600E mutation (D) in MSI-H CRC samples.

This correlation was also validated in the present study. Interestingly, we demonstrated that MS status mediated the correlation between the RNF43 frameshift mutation and these clinicopathologic variables by the confounding effect. After controlling for the MS effect by only included MSI-H patients, the I^2 declined dramatically from 67% to 0% for location, from 95% to 0% for distant metastasis, from 84% to 64% for BRAF V600E mutation and from 16% to %7 for TNM stage. These results suggest that MS status is the main cause of heterogeneity for these dataset and controlling for the MS effect is indispensable for analysing the relationship between RNF43 frameshift mutation and these

Table 2
Sensitivity analysis for relationship between RNF43 frameshift mutations and clinicopathologic characteristics.

Variable	Excluded Datasets	OR [95% CI]	I^2%	Q-test P
Site				
	Local samples	2.40[1.29-4.46]	0	0.87
	TCGA	2.58[1.42-4.69]	0	0.81
	DFCI_2016	2.57[1.46-4.52]	0	0.81
	MSK_2018	2.78[1.43-5.40]	0	0.83
	NG_2014	2.41[1.38-4.23]	0	0.95
	GUT_2017	2.84[1.54-5.25]	0	0.89
Distance metastasis				
	Local samples	1.83[0.84-4.00]	0	0.47
	TCGA	1.27[0.54-3.01]	6	0.36
	DFCI_2016	1.72[0.78-3.80]	11	0.34
	MSK_2018	1.12[0.41-3.05]	4	0.37
	NG_2014	1.80[0.83-3.87]	0	0.48
TNM				
	Local samples	0.92[0.51-1.65]	1	0.39
	TCGA	1.26[0.70-2.28]	0	1
	DFCI_2016	0.94[0.53-1.68]	5	0.37
	MSK_2018	0.85[0.44-1.65]	0	0.42
	NG_2014	0.97[0.56-1.82]	7	0.36
BRAF V600E status				
	Local samples	2.04[1.25-3.35]	44	0.13
	TCGA	1.68[0.92-2.86]	36	0.18
	DFCI_2016	2.57[1.50-4.42]	0	0.48
	MSK_2018	1.69[1.00-2.87]	35	0.19
	NG_2014	2.09[1.27-3.42]	40	0.15
	GUT_2017	1.75[1.06-2.87]	35	0.19

clinicopathologic variables. Intriguingly, After controlling for the MS effect we found that there were remaining correlations between the RNF43 frameshift mutation and both the tumour location and BRAF V600E mutation; however, no statistically significant correlation was found for the RNF43 frameshift mutation with distant metastasis and TNM stage. Jo et al. have reported that RNF43 frameshift mutation was not statistically related to TNM stage in MSI-H colorectal cancer, which was consisted with our results. Furthermore, RNF43 frameshift mutation was confirmed as a risk factor for right-sided colon cancer but was not associated with BRAF V600E mutation after sensitivity analysis.

Colorectal cancer has been classified by the MS status (MSI or MSS), chromosomal instability status (CIN) or DNA CpG island methylation status [25,26]. Different types of statuses were correlated with various tumour origins and prognoses [27,28]. The 2017 NCCN colorectal cancer guideline also notes that the sidedness of the primary lesion is correlated with drug responses, progression-free survival (PFS) and overall survival (OS) [29]. Right-sided sporadic colon cancer differs significantly in molecular characteristics that are associated with poor prognosis, with the exception of MSI-H tumours [30]. The RNF43 frameshift mutation, which acts as an independent risk factor correlated with right-sided colorectal cancer, might be considered one of the factors that contributed to the sidedness phenomenon of colorectal cancer. To our knowledge, our work revealed the association between RNF43 frameshift mutation and the right primary lesion side for the first time. The mechanisms behind the association, however, remain unknown. Das et al. reviewed promising drugs and targeted agents for colon cancer, and they determined that patients with right-sided tumour had worse results for anti-epidermal growth factor receptor (EGFR)-directed therapy than those who received bevacizumab [31]. As RNF43 is an important negative regulating molecule of the Wnt pathway, RNF43 frameshift mutation could activate Wnt pathway, which upregulates VEGF and promotes angiogenesis in colorectal cancer [32,33]. Therefore, RNF43 frameshift mutation status might partly contribute to the effect of anti-VEGF antibody bevacizumab on right-sided colon caner. Furthermore, we speculated that bevacizumab could also benefit the patients with RNF43 frameshift mutation. Otherwise, development of anti-Wnt signalling therapeutics is also necessary for the RNF43 mutant type of colon cancer [34]. The MSI-H population is particularly sensitive to the immune checkpoint inhibitors [35]. Therefore it should be further investigated whether the immune checkpoint inhibitors would also be effective for colorectal cancer patients with RNF43 frameshift mutation. If the roles of RNF43 in carcinogenesis are clarified in the future, it can be considered promising as both a predictive molecular marker and a target for intervening CRC.

4.1. Conclusion

In summary, RNF43 frameshift mutations were related to distant metastasis and TNM stage in an MS status-dependent manner. MSI-H contributes to distant metastasis and TNM stages. However, RNF43 acted as an independent risk factor that contributed to tumourigenesis in right-sided colon cancer after controlling for the MS status.

Authorship

Conceptualization, H.Z.,C.L. and W.S.; Methodology, C.L., W.S., X.W. X.X. M.L.; Investigation, D.H., E.X., M.L.; Writing–Original Draft, C.L., W.S. and H.Z.; Writing–Review & Editing, H.Z. and M.L.; Supervision, H.Z.; Funding Acquisition, C.L., H.Z. and M.L.

Competing interests

The authors disclose no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.prp.2019.152453.

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