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The Clinical Relevance of Frequent Germline Genetic Variants Detected by Targeted Sequencing in Patients With Rectal Adenocarcinoma (READ)

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Abstract. *Background: The progression of colorectal cancer (CRC) mainly stems from the occurrence of somatic mutation. However, there is little information that can be used to comprehensively analyse the importance of germline variants in CRC patients. Patients and Methods: The candidate germline variants between tumor relapse and cured rectal adenocarcinoma (READ) were firstly filtered by whole-exome sequencing (n=4), and validated by targeted sequencing and associated with clinical outcome in READ (n=48). Results: We identified 9 pathogenic germline variants that were clinically associated with survival outcome in READ, including TIPIN, TLR1, TLR10, OR4D6, IGSF3, UBBP4, OR6J1, FAM208A and DISC1. Patients carrying these germline susceptibility variants had an increased risk of poor survival outcome compared to those without these variants. Conclusion: Not only the tumor genome, but also the germline sequence must be analysed to depict the overall genetic profile, providing potential therapeutic strategies for personalized medicine.*

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Colorectal cancer (CRC) is the leading malignancy of cancer-related deaths worldwide (1), and ~30% of CRC cases are rectal adenocarcinomas (READ) (2). The standard therapeutic strategy for locally advanced rectal adenocarcinoma (LARC) is preoperative (neoadjuvant) chemoradiotherapy (neoCRT), which has been reported to control local disease and reduce distant metastasis (3-5). However, a diverse range of pathologic responses was achieved in rectal adenocarcinoma patients treated with neoCRT (6), suggesting that tumor heterogeneity affected by genetic and epigenetic variations may influence the therapeutic efficacy of neoCRT in patients with locally advanced rectal adenocarcinoma (7).

Cancer susceptibility genes affect cancer formation *via* different signalling pathways that impact the responses to treatments and the immunosurveillance of cancer cells (8). Therefore, genomic medicine has been well accepted for the diagnosis and treatment of certain types of malignancies such as chronic myeloid leukaemia (9), lung cancer (10) and colorectal cancer (11). These critical genetic mutations determine the therapeutic efficacy; therefore, detecting oncogenic mutations has become standard practice before cancer treatment. Commercial targeted sequencing cancer panels have been developed to identify somatic mutations that can be clinically actionable (12, 13), including mutations

at codon 12/13 of RAS (14), mutations at codon 600 of BRAF (15), mutations of PIK3CA, microsatellite instability (MSI) and others (11, 16). These genetic profiles significantly influence the therapeutic strategies, such as EGFR-targeted therapeutic antibody cetuximab, used for CRC patients (17). *KRAS* mutations adversely affect patients' responses to cetuximab modalities. Furthermore, mutations in *EGFR* may lead to unresponsive to cetuximab. Mutations in the downstream of EGFR signalling genes such as *PIK3CA* or *BRAF* may also adversely affect treatment response (18). Moreover, genetic polymorphisms such as *UGT1A1* have been reported to be associated with excess toxicity of the chemotherapeutic drug irinotecan (19), which suggests that germline mutations may contribute to cancer susceptibility. However, there is still no comprehensive analysis on the correlation between germline variants and cancer susceptibility, treatment responses and drug-related toxicity in rectal adenocarcinoma. It is important to identify the individual underlying variations that can potentially affect treatment to improve precision medicine and personalized oncology (13).

Herein, we utilized germline whole-exome sequencing (WES) to identify germline susceptibility variants, and validated these germline variants that may impact clinical outcome in rectal adenocarcinoma by targeted sequencing. Based on the results of WES, we comprehensive investigated the mutational status of 400 variants in 183 genes in a cohort of rectal adenocarcinoma cases. The results will help us understand the genetic background, providing information for clinical implementation in rectal adenocarcinoma.

Patients and Methods

Tumor specimen for whole-exome sequencing (WES). Our study involved 4 rectal adenocarcinoma patients who received tumor resection at the Department of Colorectal Cancer Surgery, China Medical University Hospital with written informed consent that approved by Institutional Research Board of China Medical University Hospital (CMUH105-REC2-072, Taichung, Taiwan, ROC). For whole exome sequencing, tumor tissues were stained with haematoxylin and eosin, and the selected tumor region was confirmed to be >70% tumor cell population by pathologist as previously described (20, 21). For each tumor tissue, matched normal peritumoural tissue sample was taken as a reference.

Genomic DNA (gDNA) from tumor and matched normal peritumoural samples was extracted by QIAamp DNA Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's manual. Total DNA then was used to generate genomic DNA libraries with a custom targeted capture kit designed by the Agilent SureSelect Human All Exon V5 Kit (Agilent Technologies, Santa Clara, CA, USA) (22).

Exome sequencing was performed on gDNA from tumor and matched normal peritumoural samples. The amplified DNA libraries were sequenced and analyzed by the Illumina HiSeq X Ten Genome Analyzer (Illumina, San Diego, CA, USA), yielding 150 bp of paired-end sequence reads.

The sequencing reads of gDNA were then aligned to the human genome (GRCh37) and subsequently realigned by the Genome Analysis Toolkit (GATK) to correct the individual genomes with respect to the reference genome. The final BAM files were used for variant calling, which carried out by a series of published software tools in the single nucleotide polymorphism database (dbSNP138). All variants in coding regions were visually inspected using the Integrative Genomics Viewer (IGV) for validation.

Polymorphism validation by targeted sequencing. Forty-eight rectal cancer patients with biopsy-proven tumors were collected at the China Medical University Hospital (CMUH) in the study cohort (23). From resected tumors and paired non-tumor tissues DNA and enriched DNA was extracted, by a custom QIAseq Targeted DNA Panel (cat# CDHS-12889Z-347), a multiplex PCR target enrichment panel for relevant target genes. The DNA panel was designed using the Qiagen QIAseq designer website (https://www.qiagen.com/us/shop/genes-and-pathways/custom-products/custom-array-products/qiaseq-targeted-dna-panels/#design). The PCR-amplified 400~500 bp fragments were excised in the process of gel extraction and purified using a QIAquick Gel Extraction Kit (Qiagen). No negative control PCR product existed. The prepared library was validated on an Agilent 2100 Bioanalyzer and a real-time PCR system before loading on to the up-to-date NGS sequencer, NextSeq500 (Illumina). The sequences of 2×150 bp paired-end reads were produced from the sequencer following the instructions of the manufacturer.

Statistical analysis. JMP Pro statistical software version 12 (SAS Institute, NC, USA) was used to perform the statistical analysis, including Student's *t*-test, Pearson's Chi-square test and Fisher's exact test. All statistical analysis reported a two-sided p-value <0.05 as a significance level. Cox regression analysis was utilized for univariate and multivariate models to estimate the hazard ratios (HRs) and 95% confidence intervals (CIs) (24, 25). The Kaplan-Meier survival analysis (KM curve) was used to estimate the five-year OS and DFS. The survival times were defined based on the period between surgery and events such as tumor relapse and death. The univariate comparison for survival analysis was performed using the log-rank test.

Results

Potential genomic variants were identified in rectal adenocarcinoma by whole exome sequence (WES). To identify the candidate variants that could be clinically associated with tumor relapse and survival outcome, we selected rectal adenocarcinoma patients with tumor relapse within 2 years (tumor relapse group) and patients with cured cancer (reference group) after neoCRT treatment and surgery. Then, tumor and non-tumor genomic DNA was extracted from FFPE samples for WES (Figure 1A and Table I). In terms of dbSNP annotation, we compared these two groups and identified 632 genetic variants (SNVs and small indels) that were observed only in the tumor relapse group (Figure 1B). These findings highlight that these genetic variants may be associated with tumor relapse in rectal adenocarcinoma after treatment.

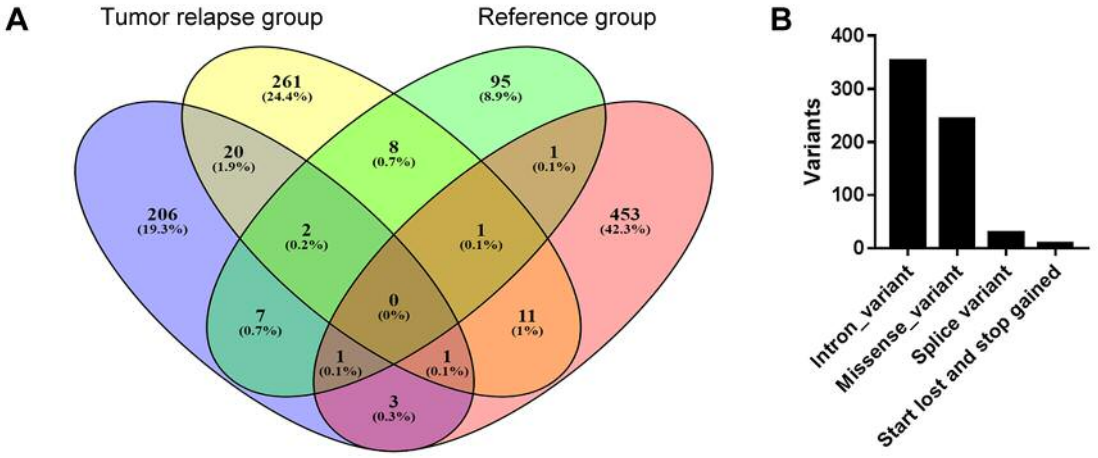

Figure 1. *WES analysis identified genetic variants that were associated with tumor relapse in rectal adenocarcinoma patients. (A) Venn diagram of unique and shared genetic variants between tumor relapse (tumor relapse group) and cured patients (reference group). (B) The unique variants were identified in tumor relapse patients.*

Table I. *The clinicopathological characters of rectal adenocarcinoma for WES (N=4).*

	Gender	Age	cT stage	cN stage	cM stage	Clinical stage (7^{th} AJCC)	pT stage	pN stage	Pathological stage (7^{th} AJCC)	Perineural invasion (PNI)	Lympho-vascular invasion (LVI)	Local recurrence (LR)	Time to LR (days)	Distant metastasis (DM)	Time to DM (days)
Tumor relapse #1	Male	53	3	0	0	IIA	2	2A	IIIB	No	No	Yes	80	Yes	616
Tumor relapse #2	Male	81	3	0	0	IIA	3	1C	IIIB	Yes	Yes	Yes	260	Yes	452
Cured #1	Female	53	3	0	0	IIA	2	0	I	No	No	No	NA	No	NA
Cured #2	Female	46	3	0	0	IIA	1	0	I	No	No	No	NA	No	NA

Clinical characteristics and survival outcome in rectal adenocarcinoma patients. To validate that these genetic variants are associated with tumor relapse in rectal adenocarcinoma, a cancer hotspot panel, the Custom QIAseq Targeted DNA Panel, was utilized to screen 48 archival FFPE samples obtained from rectal adenocarcinoma patients (Table II). This targeted DNA panel includes the immune-related genes and genetic variants that were identified by the WES. Table II presents the clinical pathological characteristics of the rectal adenocarcinoma patients (n=48). The majority of patients were female (68.8%). Twenty-four patients (50%) received pre-operative concurrent chemoradiotherapy, and 24 patients received post-operative concurrent chemotherapy. Lymphovascular invasion (LVI) was observed in 24 rectal cancer patients, and perineural invasion (PNI) was observed in 18 rectal cancer patients (26).

With this cancer hotspot panel, we screened 183 genes (347 primer fragments), and the variants were filtered based on coverage levels above 40x and p-value <0.05 to exclude the common variants, and these hotspot mutations were identified in 48 cases at variable frequency (Table III). Frequent somatic mutations were identified in RAS (p.G12V, p.G12S, p.G12D, p.G13D, p.A59T, p.E61H, p.K117N, p.A146T) and BRAF (p.V600E). Other germline variants included TIPIN (rs2063690, c.332C>G, p.A111G), TLR1 (rs4833095, c.743A>G, p.N248S), IGSF3 (rs138851517, c.1916T>C, p.I639T), OR4D6 (rs1453541, c.788T>C, p.M623T), OR6J1 (rs1753430, c.748C>T, p.P250S), SNX31 (rs2248609, c.926A>G, p.E309R), TLR10 (rs4129009, c.2323A>G, p.I775L), UBBP4 (rs72842072, c.5G>A, p.R2E), DISC1 (rs3738401, c.791G>A, p.R264E), and FAM208A (rs2291498, c.4120A>G, p.I1374V).

Genetic variants were clinically associated with 5-year DFS and 5-year OS in rectal adenocarcinoma. In these 48 rectal cancer patients, the variants were identified at variable frequencies (Figure 2A and Table IV): RAS (39.6%), TIPIN (41.7%), TLR1 (31.2%), IGSF3 (6.3%), OR4D6 (35.4%),

Table II. *Clinicopathological parameters in this study (n=48).*

Clinicopathological parameters	Total cases (%)
	48
Age	
<65	30 (62.5%)
≥65	18 (37.55)
Gender	
Female	33 (68.8%)
Male	15 (31.2%)
Clinical TNM stage (7th AJCC)	
I	4 (8.3%)
II	35 (72.9%)
III	5 (10.5%)
IV	4 (8.3%)
pT stage	
0-2	4 (8.3%)
3-4	44 (91.7%)
pN stage	
Negative	25 (52.1%)
Positive	23 (47.9%)
Pathologic TNM stage (7th AJCC)	
I	3 (6.3%)
II	22 (45.8%)
III	20 (41.6%)
IV	3 (6.3%)
Preoperative CRT	
Yes	24 (50%)
No	24 (50%)
Histological grade	
Well	2 (4.1%)
Moderate	44 (91.8%)
Poor	2 (4.1%)
LVI	
Absent	24 (50%)
Present	24 (50%)
PNI	
Absent	30 (62.5%)
Present	18 (37.5%)

LVI: Lymphovascular invasion; PNI: perineural invasion.

Table III. *Variants detected by the cancer hotspot panel in rectal adenocarcinoma.*

Gene	Mutations detected
KRAS	p.Gly12Val, p.Gly12Ser, p.Gly12Asp, p.Gly13Asp, p.A59T, p.Gln61His, p.K117N, p.Ala146Thr
NRAS	p.Gly12Val, p.Gly12Ser, p.Gly12Asp, p.Gly13Asp, p.A59T, p.Gln61His, p.K117N, p.Ala146Thr
PIK3CA	p.Glu542Lys, p.Glu545Asp, p.Glu545Gly, p.Glu545Lys, p.Gln546His, p.His1047Arg
BRAF	p.Val600Glu
TIPIN	p.A111G
TLR1	p.S602I, p.N248S
TTF1	p.A290S
IGLV3-21	p.V66I, p.D68Y
IGSF3	p.I639T
KIR2DS4	p.K156R
OR4D6	p.M263T
OR6J1	p.P250S
SNX31	p.E309R
TLR10	p.I775L
TLR3	p.L412F
TLR5	p.F616L
TLR8	p.M1V
TMPRSS11A	p.M1I
UBBp4	p.R2E
FPR1	p.E346A
DISC1	p.R264E
FAM208A	p.I374V

OR6J1 (10.4%), SNX31 (12.5%), TLR10 (33.3%), UBBp4 (4.2%), DISC1 (35.4%) and FAM208A (6.3%). In terms of disease-free survival (DFS) and overall survival (OS), rectal adenocarcinoma patients with tumors harbouring RAS somatic mutations had shorter DFS (Kaplan–Meier log-rank test, 63.8% *vs*. 39.4%, p=0.0533) and poorer OS (74.5% *vs*. 45%, p=0.0111) than patients without these mutations. Moreover, multiple somatic mutations in RAS, BRAF and PIK3CA were associated with worse 5-year DFS (67.9% *vs*. 38.4%, p=0.0308, Figure 2B) and 5-year OS (79.8% *vs*. 43.6%, p=0.0028) of patients.

Similarly, patients with germline mutations had shorter DFS and OS than patients without mutations. IGSF3-I639T variants were associated with poorer 5-year DFS (58.5% *vs*. 0.0%, p<0.0001, Figure 2C) and 5-year OS (68.1% *vs*. 1.3%, p<0.0001). TLR10-I775L variants were associated with poorer 5-year DFS (63.8% *vs*. 36.3%, p=0.0465, Figure 2D) and 5-year OS (77.2% *vs*. 39.1%, p=0.0059). UBBp4-R2E variants were associated with poorer 5-year DFS (57.2% *vs*. 1.4%, p=0.0044, Figure 2E) and 5-year OS (66.9% *vs*. 0.0%, p=0.0003). FAM208A-I1374V variants were associated with poorer 5-year DFS (58.3% *vs*. 3.6%, p=0.0055) and 5-year OS (68.0% *vs*. 7.5%, p=0.0004).

The variant OR6J1-P250S (58.8% *vs*. 16.6%, p=0.0138) was correlated with poor 5-year DFS. Some variants, such as TIPIN-A111G (76.8% *vs*. 45.3%, p=0.0125), TLR1-N248S (75.1% *vs*. 40.7%, p=0.025), OR4D6-M263T (82.4% *vs*. 52.9%, p=0.0274), SNX31-E309R (68.7% *vs*. 27.8%, p=0.0366), and DISC1-R264E (74.0% *vs*. 44.5%, p=0.0253), were significantly correlated with poor 5-year OS. Taken together, these results indicate that several germline mutations were significantly associated with 5-year survival in rectal adenocarcinoma.

Prognostic value of these variants on survival outcome of rectal adenocarcinoma patients. We then evaluated the risk factors for 5-year DFS and 5-year OS by univariate analysis. We found that patients with LVI, PNI, or variants of RAS/BRAF, IGSF3-I639, OR6J1-P250 and UBBp4-R2 had

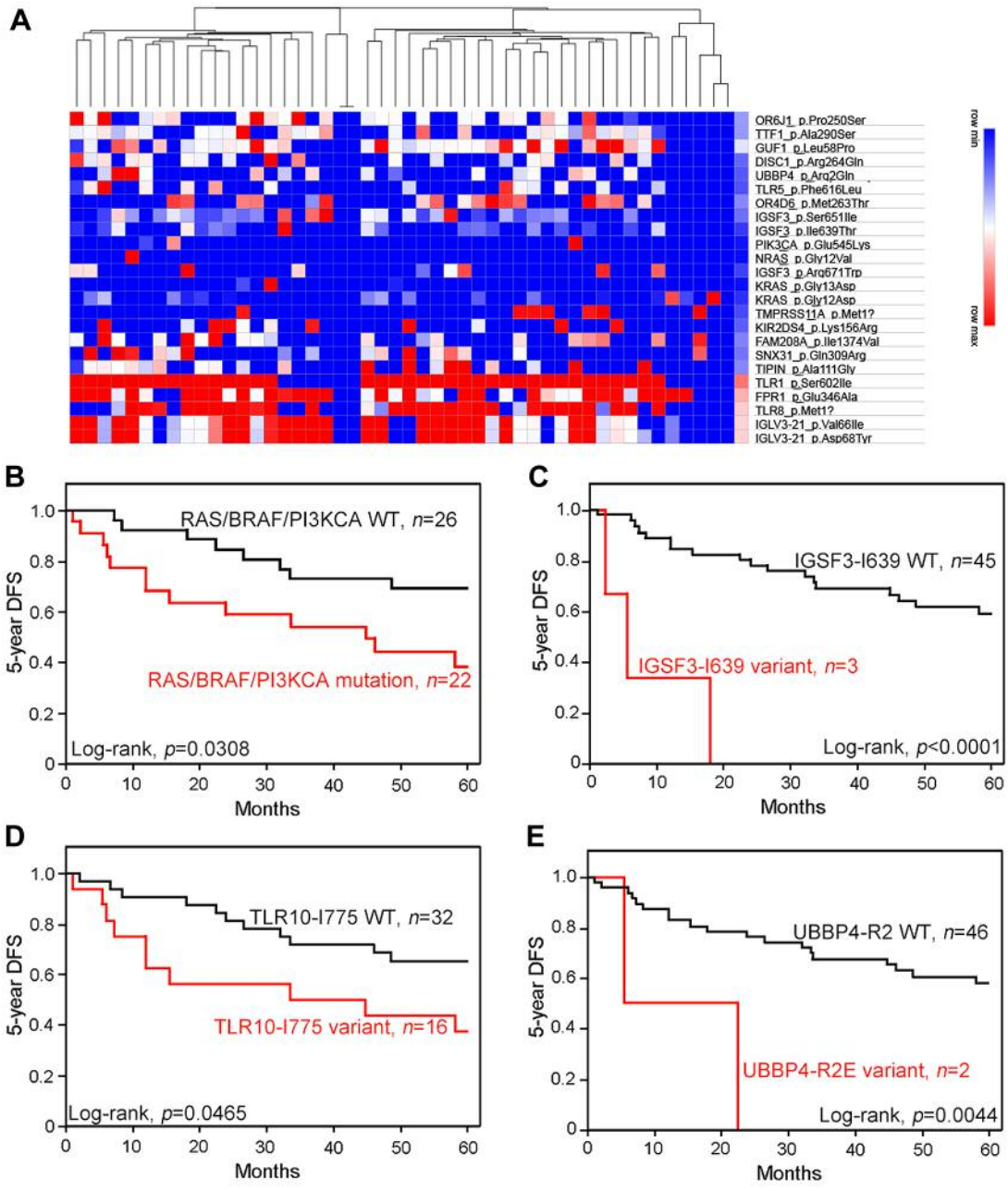

Figure 2. *Targeted sequencing identidied several gemeline variants that were clinically associated with 5-year disease-free survival in rectal adenocarcinoma (n=48). (A) Heatmap of germline variants in rectal adenocarcinoma (n=48). (B) Somatic mutations in RAS, BRAF and PI3KCA are clinically associated with 5-year DFS in READ (p=0.038, n=48). (C) Germline variant of IGSF3-I639 is associated with 5-year DFS in READ (p<0.001, n=48). (D) Germline variant of TLR10-I775 is associated with 5-year DFS in READ (p=0.0465, n=48). (E) Germline variant of UBBP4-R2 is associated with 5-year DFS in READ (p=0.0044, n=48).*

a statistically increased risk of 5-year DFS compared with the other subgroup patients (Table V). Moreover, patients with variants of RAS/BRAF, TIPIN-A111, TLR1-N248, IGSF3- I639, OR4D6-M263, TLR10-I775L, UBBP4-R2, DISC1-R264 or FAM208A-I1374 had a statistically increased risk of 5-year OS (Table V).

We then included these parameters in a multivariate Cox regression analysis to clarify independent prognostic factors

Table IV. *The association between somatic mutations, 5-year DFS and 5-year OS (n=48).*

Gene	SNP	Mutation site (a.a.)	Total cases (%)		5-year DFS			5-year OS		
			WT (%)	Variant (%)	WT survival (%)	Variant survival (%)	*p*-Value	WT survival (%)	Variant survival (%)	*p*-Value
RAS			29 (60.4%)	19 (39.6%)	63.8%	39.4%	0.0533	74.5%	45.0%	0.0111
RAS/BRAF			27 (56.3%)	21 (43.7%)	69.1%	35.4%	0.0139	80.5%	40.9%	0.0012
RAS/BRAF/PIK3CA			26 (54.2%)	22 45.8%)	67.9%	38.4%	0.0308	79.8%	43.6%	0.0028
TIPIN	rs2063690 (c.332C>G)	A111G	28 58.3%)	20 (41.7%)	61.9%	44.3%	0.171	76.8%	45.3%	0.0125
TLR1	rs5743618 (c.1805G>T)	S602I	11 (22.9%)	37(77.1%)	39.9%	58.6%	0.2436	62.0%	64.4%	0.8343
TLR1	rs4833095 (c.743A>G)	N248S	33 68.8%)	15 (31.2%)	62.3%	37.9%	0.1377	75.1%	40.7%	0.025
TTF1	rs8999 (c.868G>T)	A290S	21 (43.8%)	27 (56.3%)	64.3%	46.3%	0.1476	73.8%	55.1%	0.113
IGLV3-21	rs4446155 (c.196G>A)	V66I	12 (25%)	36 (75%)	73.0%	48.3%	0.1385	69.3%	60.4%	0.3462
IGLV3-21	rs1985791 (c.202G>T)	D68Y	11 (22.9%)	37 (77.1%)	70.3%	49.7%	0.2157	65.8%	61.5%	0.4754
IGSF3	rs138851517 (c.1916T>C)	I639T	45 (93.8%)	3 (6.3%)	58.5%	0.0%	<0.0001	68.1%	1.3%	<0.0001
KIR2DS4	rs4806590 (c.467A>G)	K156R	37 (77.1%)	11 (22.9%)	60.6%	34.6%	0.129	66.9%	53.7%	0.3487
OR4D6	rs1453541 (c.788T>C)	M263T	31 (64.6%)	17 (35.4%)	48.7%	64.3%	0.2437	82.4%	52.9%	0.0274
OR6J1	rs1753430 (c.748C>T)	P250S	43 (89.6%)	5 (10.4%)	58.8%	16.6%	0.0138	66.4%	36.4%	0.0721
SNX31	rs2248609 (c.926A>G)	E309R	42 (87.5%)	6 (12.5%)	58.2%	27.5%	0.0989	68.7%	27.8%	0.0366
TLR10	rs4129009 (c.2323A>G)	I775L	32 (66.7%)	16 (33.3%)	63.8%	36.3%	0.0465	77.2%	39.1%	0.0059
TLR3	rs3775291 (c.1234C>T)	L412F	26 (54.2%)	22 (45.8%)	46.6%	63.3%	0.2688	60.0%	68.1%	0.5618
TLR5	rs5744174 (c.1846T>C)	F616L	34 (70.8%)	14 (29.2%)	57.3%	47.4%	0.4681	69.9%	48.9%	0.1325
TLR8	rs3764880 (c.1A>G)	M1V	16 (33.3%)	32 (66.7%)	40.2%	61.2%	0.1106	54.6%	67.6%	0.239
TMPRSS11A	rs977728 (c.3G>A)	M1I	41 (85.4%)	7 (14.6%)	86.5%	47.4%	0.0909	60.0%	86.5%	0.1935
UBBp4	rs72842072 (c.5G>A)	R2E	46 (95.8%)	2 (4.2%)	57.2%	1.4%	0.0044	66.9%	0.0%	0.0003
FPR1	rs867228 (c.1037A>T)	E346A	23 (47.9%)	25 (52.1%)	63.1%	46.0%	0.1676	72.0%	55.1%	0.1548
DISC1	rs3738401 (c.791G>A)	R264E	31 (64.6%)	17 (35.4%)	63.5%	37.3%	0.0568	74.0%	44.5%	0.0253
FAM208A	rs2291498 (c.4120A>G)	I374V	45 (93.8%)	3 (6.3%)	58.3%	3.6%	0.0055	68.0%	7.5%	0.0004

The survival outcomes were estimated by Kaplan-Meier survival analysis. RAS variants are defined as significant mutations in KRAS/NRAS gene. BRAF variant is defined as V600E mutation. PIK3CA variants is defined as significant mutations in *PIK3CA* gene.

for rectal adenocarcinoma (Table VI). Our results showed that the OR6J1-P250 variant (HR=5.638, 95% CI=1.379-19.816, p=0.0185) and IGSF3-I639 variant (HR=9.583, 95% CI=1.286-81.499, p=0.0283) were independent prognostic factors for 5-year DFS, and the TIPIN-A111 (HR=6.356, 95% CI=1.02-63.554, p=0.0473), IGSF3-I639 (HR=74.518, 95% CI=4.434-2373.298, p=0.0023), OR4D6-M263 (HR=34.479, 95% CI=3.306-727.873, p=0.0016) and DISC1-R264 (HR=5.622, 95% CI=1.171-35.822, p=0.0304) variants were independent prognostic factors for 5-year OS for rectal adenocarcinoma (Table VI). These data strongly indicate that these polymorphisms have significant clinical prognostic value for rectal adenocarcinoma.

Discussion

With the advance of NGS technologies, genetic variants have been gradually revealed to be associated with disease occurrence. Herein, we demonstrated the application of WES and a targeted sequence panel to identify the impact of germline genetic variants on clinical outcomes. Our results highlight that germline variants affect survival outcome and tumor relapse, which suggested that screening for the variants might be taken into consideration after treatment of rectal adenocarcinoma patients.

By comprehensive analysis of the targeted sequence panel and clinical database, we validated 9 germline variants that were significantly associated with 5-year DFS and 5-year OS. Several genetic variants were associated with cancer susceptibility and have been extensively reported. For example, TLR1-N248S (rs4833095) is associated with an increased risk of gastric cancer in a European population (27), IGSF3 variants are associated with an increased risk of hepatocarcinoma, ovarian cancer and colorectal cancer (28) and the TLR10-I775 (rs4129009) variant is associated with bladder cancer (29). Moreover, our study showed that several variants that have been implicated in disease occurrence are correlated with survival outcome in rectal adenocarcinoma. These results suggest that variants such as TIPIN-A111G (rs2063690), OR4D6-M263T (rs1453541), DISC1-I639T (rs3738401) and FAM208A-I1374V (rs2291498) might be cancer-related variants. Furthermore, we identified two novel

Table V. *Univariate analysis of somatic mutations, 5-year DFS and 5-year OS (n=48).*

Gene	5-year DFS			5-year OS		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
Gender (Male *vs*. Female)	1.169	0.475-3.282	0.744	1.204	0.446-3.789	0.7237
Age (>65 *vs*. <65)	1.569	0.639-3.714	0.3153	1.796	0.673-4.71	0.2351
cN stage (Positive *vs*. Negative)	1.767	0.747-4.333	0.1945	2.581	0.980-7.501	0.055
pN stage (Positive *vs*. Negative)	2.04	0.858-5.163	0.1071	2.405	0.913-6.994	0.0762
LVI (Present *vs*. Absent)	3.339	1.349-9.407	0.0085	3.145	1.161-9.921	0.0237
PNI (Present *vs*. Absent)	3.113	1.313-7.65	0.0103	7.943	2.792-28.327	<0.0001
RAS (Variant *vs*. WT)	2.278	0.062-05.476	0.062	3.285	1.255-9.091	0.0157
RAS/BRAF (Variants *vs*. WT)	2.887	1.213-7.312	0.0166	4.833	1.777-15.294	0.0018
TIPIN (Variant *vs*. WT)	1.805	0.76-4.337	0.1781	3.311	1.258-9.627	0.0152
TLR1-S602 (Variant *vs*. WT)	0.572	0.231-1.61	0.271	0.887	0.314-3.153	0.8362
TLR1-N248 (Variant *vs*. WT)	1.907	0.777-4.517	0.1538	2.84	1.084-7.578	0.0341
TTF1 (Variant *vs*. WT)	1.933	0.803-5.106	0.1434	2.273	0.842-7.149	0.107
IGLV3-21-V66(Variant *vs*. WT)	2.446	0.827-10.447	0.1128	1.806	0.589-7.837	0.3239
IGLV3-21-D68 (Variant *vs*. WT)	2.127	0.719-9.082	0.1875	1.569	0.512-6.844	0.4583
IGSF3 (Variant *vs*. WT)	11.558	2.481-41.002	0.0041	13.012	2.741-48.146	0.0031
KIR2DS4 (Variant *vs*. WT)	1.996	0.755-4.808	0.1547	1.643	0.52-4.459	0.3711
OR4D6 (Variant *vs*. WT)	0.5723	0.204-1.412	0.2334	0.267	0.061-0.827	0.0202
OR6J1 (Variant *vs*. WT)	3.638	1.038-9.951	0.0443	2.991	0.675-9.239	0.1286
SNX31 (Variant *vs*. WT)	2.443	0.699-6.656	0.1459	3.139	0.877-8.972	0.0748
TLR10 (Variant *vs*. WT)	2.332	0.97-5.544	0.0582	3.569	1.369-9.845	0.0096
TLR3 (Variant *vs*. WT)	0.61	0.241-1.453	0.2663	0.752	0.272-1.96	0.5605
TLR5 (Variant *vs*. WT)	1.398	0.529-3.362	0.4791	2.069	0.75-5.4	0.1534
TLR8 (Variant *vs*. WT)	0.5	0.211-1.229	0.127	0.564	0.216-1.554	0.2562
TMPRSS11A (Variant *vs*. WT)	0.208	0.012-1.002	0.0504	0.284	0.015-1.397	0.1407
UBBP4 (Variant *vs*. WT)	6.767	1.034-25.919	0.0468	10.825	1.571-47.752	0.0205
FPR1 (Variant *vs*. WT)	1.843	0.776-4.664	0.167	2.031	0.771-5.905	0.153
DISC1 (Variant *vs*. WT)	2.263	0.937-5.402	0.0687	2.852	1.083-7.643	0.0342
FAM208A (Variant *vs*. WT)	4.949	1.136-15.276	0.0356	7.758	1.694-26.97	0.012

The log-rank tests were performed for univariate comparison. RAS variants are defined as significant mutations in KRAS/NRAS gene. BRAF variant is defined as V600E mutation.

Table VI. Multivariate analysis of somatic mutations in 5-year DFS and 5-year OS (n=48).

Gene	5-year DFS			5-year OS		
	HR	95% CI	*p*-Value	HR	95% CI	*p*-Value
LVI (Present *vs*. Absent)	1.829	0.593-5.891	0.292	1.666	0.218-13.009	0.618
PNI (Present *vs*. Absent)	2.346	0.763-6.902	0.1329	56.594	6.788-738.939	<0.0001
RAS/BRAF (Variants *vs*. WT)	4.322	1.685-12.054	0.0022	3.217	0.377-39.963	0.3084
TIPIN-A111 (Variant *vs*. WT)	-	-	-	6.356	1.02-63.554	0.0473
TLR1-N248 (Variant *vs*. WT)	-	-	-	3.472	0.755-19.063	0.1105
IGSF3-I639 (Variant *vs*. WT)	9.583	1.286-81.499	0.0283	74.517	4.434-2373.298	0.0023
OR4D6-M263 (Variant *vs*. WT)	-	-	-	34.479	3.306-727.873	0.0016
UBBP4-R2 (Variant *vs*. WT)	3.124	0.223-24.489	0.3638	0.129	0.003-3.317	0.2216
FAM208A-I374 (Variant *vs*. WT)	1.165	0.16-5.821	0.8641	0.504	0.037-4.85	0.5639
OR6J1-P250 (Variant *vs*. WT)	5.638	1.379-19.816	0.0185	-	-	-
TLR10-I775L (Variant *vs*. WT)	-	-	-	1.412	0.149-11.915	0.7515
DISC1-R264 (Variant *vs*. WT)	-	-	-	5.622	1.171-35.822	0.0304

The Cox regression tests were performed for multivariate comparison. RAS variants are defined as significant mutations in K-RAS/N-RAS gene. BRAF variant is defined as V600E mutation.

variants, SNX31-E309R (rs2248609) and UBBp4-R2E (rs72842072), that were correlated with poor 5-year DFS and 5-year OS. Taken together, our results suggest that these genetic variants are potential risk factors for tumor relapse and poor survival outcome in rectal adenocarcinoma. Importantly, by comparing to the pathogenic cancer-associated variants in the ClinVar database, we identified only two germline variants TLR1-N248S (rs4833095) and DISC1-I639T (rs3738401) have reported a relatively high frequency in diseases. These results indicated that patients with germline genetic variants, previously identified as non-oncogenic impacts should be carefully monitored. Therefore, precision oncology by NGS or targeted sequencing panel for germline variants seems to be a promising strategy for patients. But there are limitations in current study such as small sample size and different therapeutic strategies in rectal adenocarcinoma patient. More studies are needed to confirm the prognostic value of these variants in rectal adenocarcinoma.

Taken together, our findings highlight the importance of applying comprehensive genomic information to determine therapeutic strategies for rectal adenocarcinoma. Germline sequence results and tumor genomes have to be integratively analysed to provide more information on the overall genetic background of cancer development in rectal adenocarcinoma.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

Authors' Contributions

Kevin Chih-Yang Huang and Shu-Fen Chiang conducted and performed the experiments. William Tzu-Liang Chen, Tao-Wei Ke and Tsung-Wei Chen participated in the clinical information collection for rectal carcinoma patients. Shu-Fen Chiang performed the statistical analysis. Shu-Fen Chiang, William Tzu-Liang Chen and K. S. Clifford Chao supervised this study. Kevin Chih-Yang Huang, Shu-Fen Chiang and K. S. Clifford Chao analysed the data and wrote the manuscript.

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