Tohoku J. Exp. Med., 2022, **257**, 163-169

RECK Variants are Associated with Clinicopathological Features and Decreased Susceptibility in Mexican Patients with Colorectal Cancer

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Colorectal cancer (CRC) is the third most common cancer and the second leading cause of death worldwide. Down-regulation of the cysteine-rich reversion-inducing protein with Kazal motifs (RECK) has been confirmed in numerous human cancers and is clinically associated with metastasis. This study aims to explore, for the first time, the possible association of the *RECK* variants rs11788747 and rs10972727 with CRC susceptibility and clinicopathological features. DNA from 130 CRC patients and 130 healthy blood donors was analyzed. Identification of genetic variants was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodology. Association was calculated using the odds ratio (OR) test and P values were adjusted using the Bonferroni test. Individuals carrying the G/G genotype for the rs11788747 variant showed a lower risk of colorectal cancer (OR 0.33; 95% CI 0.16-0.70; P = 0.006). Patients older than 50 years who carry the G/G genotype have a lower risk of CRC (OR 0.26; 95% CI 0.09-0.73; P = 0.019) and of developing advanced tumor-nodule-metastasis (TNM) stages (OR 0.23; 95% CI 0.09-0.54; P = 0.001). Individuals carrying the A/A genotype of the rs10972727 variant also showed decreased risk of CRC (OR 0.38; 95% CI 0.19-0.77; P = 0.011), and were associated with age (over 50 years), sex, advanced TNM stages, and tumor location in the colon. Our results suggest that the *RECK* variants studied here (rs11788747 and rs10972727) are associated with decreased CRC risk, TNM stages and tumor location.

Keywords: colorectal cancer; decreased susceptibility; haplotypes; *RECK* variants; TNM stage; tumor location
Tohoku J. Exp. Med., 2022 June, **257** (2), 163-169.
doi: 10.1620/tjem.2022.J032

Received December 22, 2021; revised and accepted March 27, 2022; J-STAGE Advance online publication April 21, 2022

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Introduction

Colorectal cancer (CRC) is the third most frequent cancer in American countries (Sung et al. 2021). The CRC incidence in Mexico reached 14,901 cases during 2020 (11.6/100,000) and a mortality rate of 5.4/100,000 inhabitants (Globocan 2020). CRC is a complex disease caused by environmental factors, genetic, epigenetic, lifestyle, and dietary habits (Fischer et al. 2019); disease progression is regulated through interactions with multiple genes involved in proliferation, migration, invasion, angiogenesis, and metastasis. Among these, the reversion-inducing cysteine-rich protein with Kazal motifs (*RECK*) gene is located on 9p13-p12, exhibits 87 kb of length with 21 exons, and is known as a transformation suppressor (Meng et al. 2008). The RECK protein form a glycoprotein which is anchored to the plasma membrane via a COOH-terminal glycosylphosphatidylinositol modification and has a significant effect on tumorigenesis through negative regulation of the matrix metalloproteinases (MMPs), specifically of MMP-4 and MMP-9, inhibiting angiogenesis and tumor invasion through the extracellular matrix (Oh et al. 2001; Qi et al. 2010). *RECK* gene expression is found downregulated in many human cancers as breast (Span et al. 2003), pancreas (Masui et al. 2003), non-small cell lung (Takenaka et al. 2004), colorectal (Takeuchi et al. 2004), gastric (Song et al. 2006), prostate (Rabien et al. 2007), oral squamous cell carcinoma (Long et al. 2008), osteosarcoma (Xu et al. 2010) and hepatocellular carcinoma (Chung et al. 2012). Modifications in the function of the *RECK* gene have also been associated with lymph node metastases (Chang et al. 2007; Chung et al. 2012; Fakhry et al. 2016). In osteosarcoma, high expression of *RECK* contributes to reducing tumor invasion and increasing survival rates (Kang et al. 2007).

Currently, 13 single nucleotide polymorphisms (SNPs) have been identified on the *RECK* gene, four of them are located in the coding region of exons 1, 9, 13 and 15; remaining SNPs are in introns 5, 8, 10, 12, 15 and 17 (Eisenberg et al. 2002; Meng et al. 2008). Among these, rs11788747 variant located in exon 13 (c.1176 A > G or Pro520Pro) has been studied and associated with several cancer types (Chung et al. 2012; Chen et al. 2014; Yu et al. 2015; Abd-Elfatah and Gad-Allah 2016; Bahgat et al. 2016; Zhang et al. 2017) but no in CRC. On the other hand, the rs10972727 variant located in exon 15 (c.1491 T > A or Arg625Arg) has not been associated with any cancer (Chung et al. 2012; Yu et al. 2015; Abd-Elfatah and Gad-Allah 2016).

This study aims to evaluate, for the first time, the possible association of genotypes, alleles, and haplotypes of the rs11788787 and rs10972727 variants in the *RECK* gene with the development of CRC and with its clinicopathological features.

Materials and Methods

Study population

The study was approved by the Ethics Committee 1305 of the West Biomedical Research Center of the Mexican Institute of Social Security (IMSS) (R-2018-1305-001) and conducted under national and international ethical standards. All patients and control individuals signed an informed consent for participation. In this study, 130 CRC patients and 130 unrelated healthy individuals, matched in age and sex with the case group, were recruited from 2018 to 2020 at the Specialty Hospital of West National Medical Center at IMSS in Guadalajara, Mexico. The group of patients included individuals clinically diagnosed and histologically confirmed as sporadic colorectal adenocarcinoma according to the Clinical Practice Guidelines on Colon and Rectal Cancer and the clinicopathological criteria of the Specialty Hospital of West National Medical Center in the IMSS in Guadalajara, Mexico. Tumor staging and grading was performed according to the tumor-node-metastasis (TNM) classification. The control group included unrelated healthy individuals with negative colonoscopy for malignancy. Exclusion criteria for patients and control individuals included a negative diagnosis of autoimmune or inflammatory bowel disease and a family history of any known hereditary cancer syndrome. Personal data, including sex, age, smoking and drinking habits, family history, and clinical and pathologic characteristics of the patients were obtained from hospital records.

Genotyping

Genomic DNA was isolated from peripheral blood using standard methods (Miller et al. 1988). The variants rs11788747 (A > G) and rs10972727 (T > A) in the *RECK* gene were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the following primer pairs. For the variant rs11788747-F: 5´-GTA GAA GAA GTG ACT CAT CC-3´ and rs11788747-R: 5´- ATC TCA CTC CGA AGA TAA CC-3´. For the variant rs10972727-F: 5´- TTCTGT CAG GTC ATG GAA CA -3´and rs10972727-R: 5´- TGC AGT TAA GAC TGG AGA AG -3´ (Chung et al. 2012). For the rs11788747 variant, PCR was performed in a 10 μL volume containing 10× buffer (500 mM KCl, 100 mM Tris- HCl, and 0.1% Triton TMX-100), 2.0 mM $MgCl_2$, 150 μM dNTPs, 1 μM of each primer, 3 U Taq DNA polymerase and 50 ng of DNA. The PCR program used was 95°C for 5 min, followed by 35 cycles of 94°C for 4 min, annealing at 59°C for 30 sec, elongation at 72°C for 30 sec and a final extension for 5 min at 72°C. The rs10972727 variant was identified under the same PCR conditions except for the annealing temperature (58°C). Three units of RsaI enzyme restriction at 37°C overnight (New England Biolabs, Ipswich, MA, USA) were used to digest 5 μL of the PCR product of the rs11788747 variant, according to the manufacturer's instruction. The digested products were separated on 6% polyacrylamide

gels. The homozygous polymorphic genotype G/G contains a recognition site for the enzyme RsaI, so digestion of the amplification product, using RsaI, yields two DNA fragments of 140 and 102 bp in length. The homozygous wild genotype A/A does not contain a recognition site for the enzyme RsaI, so the 242 bp amplificon remains unaltered after incubation with RsaI. Incubation of the heterozygous genotype A/G with RsaI yields DNA fragments of 242, 140 and 102 bp. For the rs10972727 polymorphism, five microliters of the amplification product were digested with 3U of HpyCH4IV restriction enzyme at 37°C overnight (New England Biolabs) according to the manufacturer's instructions and separated on 6% polyacrylamide gels. The homozygous wild genotype T/T does not contain a recognition site for the enzyme HpyCH4IV, so the 224 bp amplificon remains unaltered after incubation with HpyCH4IV. The homozygous polymorphic genotype A/A contains a recognition site for the enzyme HpyCH4IV, so the digestion of the amplification product, using HpyCH4IV, yields two DNA fragments 119 and 105 bp in length. Incubation of the heterozygous genotype T/A with HpyCH4IV yields three DNA fragments 224, 119 and 105 bp. Quality control for these assays was evaluated by re-genotyping 10% of samples randomly selected by an independent technician. Concordance between genotyping tests was 100%.

Statistical analysis

Allelic and genotype frequencies were estimated by direct counting in both groups. The Chi-square test assessed the Hardy-Weinberg equilibrium (HWE). Statistical analysis included odds ratio analysis and Yates corrected Chi-square test. Association of alleles or genotypes with CRC and with demographic and clinicopathological features were calculated by odds ratio (OR) and confidence intervals (CI) in a SPSS v25.0 software package (SPSS Inc., Chicago, IL, USA). Haplotype analysis was performed using the Haploview 4.2 software. For all statistical analysis $P < 0.05$ was considered significant. A Bonferroni correction test was applied to adjust the P values ($P < 0.025$).

Results

Clinical features of the subjects included in the study

Table 1 shows the clinicopathological features of the CRC patients and the control group. The mean age observed was 49.68 (± 8.4) years for the colorectal cancer group, and 48.22 (± 6.1) years in the control group ($P = 0.109$). Stratification by age (< 50 and > 50) showed significant differences between CRC and control groups ($P = 0.001$). Alcohol and tobacco consumption also showed significant differences ($P = 0.001$ and $P = 0.015$ respectively) between these groups. Sex did not show significant differences between the groups analyzed ($P > 0.05$).

RECK variants in CRC patients and control group

Table 2 shows a comparative analysis of *RECK* variants in CRC patients and in the control group. The two variants analyzed in the control group were in HWE ($P > 0.05$). Analyses of the two *RECK* variants in CRC patients and in the control group showed significant differences. Individuals carrying the G/G genotype of the *RECK* rs11788747 variant showed a decreased risk of CRC (OR 0.33; 95% CI 0.16-0.70; $P = 0.006$). In the analysis of allele frequencies, individuals carrying the G allele showed a decreased susceptibility to CRC (OR 0.60; 95% CI 0.43-0.86; $P = 0.006$). Regarding the *RECK* rs10972727 variant, individuals with A/A genotype showed a lower susceptibility to develop CRC (OR 0.38; 95% CI 0.19-0.77; $P = 0.011$). Allele frequencies also exhibited significant differences (OR 0.62; 95% CI 0.44-0.88; $P = 0.010$) showing that the A allele was less frequent among CRC patients. Four different haplotypes were observed in the *RECK* gene, although none were associated with the CRC.

RECK genotypes by sex, age, TNM stage and tumor localization

Association analysis with demographic and clinicopathological features for each *RECK* variant is shown in Tables 3 and 4. For the rs11788747 variant, a significant difference was observed between the CRC and control groups in advanced age (> 50 years) (OR 0.26; 95% CI 0.09-0.73; $P = 0.019$); decreased G/G vs. A/A genotypes was also observed in CRC patients with advanced TNM stages (IV and III + IV) (OR 0.23; 95% CI 0.09-0.54; $P = 0.001$ and OR 0.19; 95% CI 0.06-0.60; $P = 0.006$), respectively (Table 3).

For the rs10972727 variant, the genotype homozygote polymorphic (A/A) was significantly decreased in CRC males (OR 0.19; 95% CI 0.06-0.56; $P = 0.004$). Such a difference was also observed in CRC patients with advanced age (> 50 years) (OR 0.28; 95% CI 0.10-0.73; $P = 0.017$). In the analysis by TNM stage, we also found a significant decrease of the genotype A/A (homozygous polymorphic) in patients with CRC in advanced stages (III + IV) (OR 0.36; 95% CI 0.16-0.78; $P = 0.015$), and with tumor location in colon (OR 0.25; 95% CI 0.09-0.67; $P = 0.008$) (Table 4).

Discussion

With the results of this study, we provide novel information on the effects of *RECK* rs11788747 and rs10972727 variants on CRC risk and their association with clinicopathological features in Mexican patients.

Among the 130 CRC patients analyzed, 65.4% were older than 50 years. It is known that the risk of colorectal cancer increases as people age, although it can occur even in adolescents and young adults (Siegel et al. 2014; Ferlay et al. 2015; Cancer Network Home of the Journal Oncology 2016); however, when the genotypes of *RECK* risk variants were analyzed in individuals > 50 years, carriers of the polymorphic G/G and A/A genotypes of the rs11788747 and rs10972727 variants showed a lower risk of developing

Table 1. Demographic and clinical characteristics of the colorectal cancer (CRC) patients and control subjects.

Characteristic	CRC group n = 130 (100%)	Control group n = 130 (100%)	P value
Mean age (± SD), years	49.68 (± 8.4)	48.22 (± 6.1)	0.109
Age (in years)			
< 50	45 (34.6)	75 (57.6)	**0.001**
> 50	85 (65.4)	55 (42.4)	
Sex			
Female	60 (46.1)	67 (51.5)	0.457
Male	70 (53.9)	63 (48.5)	
Smoking status			
Yes	48 (36.9)	16 (12.3)	**0.001**
No	82 (63.1)	109 (87.7)	
Drinking status			
Yes	36 (27.7)	19 (14.6)	**0.015**
No	94 (72.3)	111 (85.4)	
Clinical stage TNM			
I	2 (1.5)		
II	39 (30.0)		
III	48 (36.9)		
IV	41 (31.6)		
Tumor location			
Colon	66 (50.8)		
Rectum	64 (49.2)		
Site of metastasis			
Liver	15 (36.6)		
Lung	9 (21.9)		
Liver and lung	6 (14.6)		
Peritoneum	2 (4.9)		
Ovary	2 (4.9)		
No date	7 (17.1)		
Treatment response			
Non-response	32 (24.6)		
Partial response	41 (31.5)		
Complete response	57 (43.9)		

Data are shown as n (%), except mean age (± SD). P values were calculated by the Chi-square test. Bold text highlights statistically significant findings.

cancer, respectively.

Regarding the rs11788747 variant, similar findings have been previously described in patients with Wilms tumor (Yu et al. 2015) and in patients with hepatocellular carcinoma (Abd-Elfatah and Gad-Allah 2016); however, other authors found an increased risk associated with this variant in oral cancer (Chung et al. 2011), hepatocellular carcinoma (Chung et al. 2012; Bahgat et al. 2016) and non-small cell lung cancer (Chen et al. 2014). With reference to TNM stage, CRC patients carrying this same G/G genotype also have a lower risk of reaching advanced TNM stage; that is, these patients appear to show some protection to progress to a more severe clinical stage. This result is similar to that described by Yu et al. (2015) in patients with Wilms tumor. We could not find a significant association between tumor location and any of the variants analyzed here.

So far, the rs11788747 variant has shown contradictory results regarding the susceptibility or risk of developing cancer in different populations studied as oral cancer in Taiwan (Chung et al. 2011); hepatocellular carcinoma in Taiwan (Chung et al. 2012); non-small cell lung cancer (NSCLC) in Chinese (Chen et al. 2014); Wilms tumor in Chinese (Yu et al. 2015); hepatocellular carcinoma in Egypt (Abd-Elfatah and Gad-Allah 2016; Bahgat et al. 2016) and Ameloblastoma in Chinese (Zhang et al. 2017).

The rs10972727 variant also showed significant differences in genotype distribution between the groups ana-

Table 2. Distribution of genotypes, allelic frequencies and haplotypes of the *RECK* rs11788747 and rs10972727 variants in colorectal cancer (CRC) and control groups.

Genotype	CRC group n = 130 (%)	Control group n = 130 (%)	OR (95% CI)	P value
RECK (rs11788747)				
A/A	40 (30.8)	25 (19.2)	1.00 (Reference)	
A/G	70 (53.8)	68 (52.3)	0.64 (0.35-1.17)	0.196
G/G	20 (15.4)	37 (28.5)	**0.33 (0.16-0.70)**	**0.006**
A/G + G/G vs. A/A	90 (69.2)	105 (80.8)	0.53 (0.30-0.95)	0.044
Allele				
A	150 (57.7)	118 (45.4)	1.00 (Reference)	
G	110 (42.3)	142 (54.6)	**0.60 (0.43-0.86)**	**0.006**
RECK (rs10972727)				
T/T	44 (33.8)	34 (26.1)	1.00 (Reference)	
A/T	66 (50.7)	56 (43.1)	0.91 (0.51-1.61)	0.861
A/A	20 (15.5)	40 (30.8)	**0.38 (0.19-0.77)**	**0.011**
A/T + A/A vs. T/T	86 (66.1)	96 (73.8)	0.69 (0.40-1.18)	0.223
Allele				
T	154 (59.2)	124 (47.7)	1.00 (Reference)	
A	106 (40.8)	136 (52.3)	**0.62 (0.44-0.88)**	**0.01**
Haplotype		*RECK* rs11788747-rs10972727		
G-T	38 (29.4)	37 (28.6)	1.03 (0.60-1.77)	1
A-A	36 (27.9)	34 (26.3)	1.08 (0.62-1.87)	0.888
A-T	39 (29.8)	25 (19.1)	1.80 (1.01-3.19)	0.061
G-A	17 (12.9)	34 (26.0)	0.52 (0.27-1.00)	0.073

Data are shown as n (%). P values were calculated by the chi-square test. Bold text highlights statistically significant findings.
OR, odds ratio; CI, confidence interval.

Table 3. Association of the *RECK* rs11788747 variant with demographic and clinical variables.

Variable	*RECK* (rs11788747)					
	CRC/Control			OR (95% CI); P value		
	AA	AG	GG	AG vs. AA	GG vs. AA	AG + GG vs. AA
Sex						
Male	22/12	40/34	8/17	0.64 (0.27-1.48); 0.406	0.25 (0.08-0.76); 0.026	0.51 (0.22-1.14); 0.151
Female	18/13	30/34	12/20	0.63 (0.26-1.51); 0.421	0.43 (0.15-1.19); 0.167	0.56 (0.24-1.27); 0.237
Age (years)						
< 50	13/15	24/40	8/20	0.69 (0.28-1.70); 0.566	0.46 (0.15-1.39); 0.269	0.61 (0.26-1.45); 0.372
> 50	27/10	46/28	12/17	0.60 (0.25-1.44); 0.357	**0.26 (0.09-0.73); 0.019**	0.47 (0.20-1.08); 0.113
TNM stage						
I + II	8/25	24/68	9/37	1.10 (0.43-2.77); 1.000	0.76 (0.25-2.23); 0.824	0.98 (0.40-2.38); 1.000
III + IV	32/25	46/68	11/37	0.52 (0.27-1.00); 0.073	**0.23 (0.09-0.54); 0.001**	**0.42 (0.22-0.78); 0.008**
III	15/25	27/68	6/37	0.66 (0.30-1.44); 0.402	0.27 (0.09-0.79); 0.026	0.52 (0.24-1.10); 0.132
IV	17/25	19/68	5/37	0.41 (0.18-0.91); 0.045	**0.19 (0.06-0.60); 0.006**	**0.33 (0.15-0.71); 0.007**
Localization						
Colon	18/25	38/68	8/37	0.77 (0.37-1.60); 0.617	0.30 (0.11-0.79); 0.024	0.60 (0.30-1.22); 0.223
Rectum	22/25	32/68	12/37	0.53 (0.26-1.08); 0.120	0.36 (0.15-0.87); 0.038	0.47 (0.24-0.93); 0.044

P values were adjusted by the Bonferroni test (0.025). Bold text highlights statistically significant findings.
OR, odds ratio; CI, confidence interval.

Table 4. Association of the *RECK* rs10972727 variant with demographic and clinical variables.

Variable	*RECK* (rs10972727)					
	CRC/Control			OR (95% CI); P value		
	TT	TA	AA	TA vs. TT	AA vs. TT	TA + AA vs. TT
Sex						
Male	25/13	37/29	8/21	0.66 (0.28-1.51); 0.443	**0.19 (0.06-0.56); 0.004**	0.46 (0.21-1.02); 0.083
Female	19/21	29/27	12/19	1.18 (0.52-2.67); 0.835	0.69 (0.26-1.80); 0.617	0.98 (0.46-2.08); 1.000
Age (years)						
< 50	11/22	28/31	6/22	1.80 (0.74-4.38); 0.273	0.54 (0.17-1.73); 0.455	1.43 (0.62-3.30); 0.522
> 50	Dec-33	38/25	14/18	0.55 (0.24-1.26); 0.230	**0.28 (0.10-0.73); 0.017**	0.43 (0.20-0.95); 0.055
TNM stage						
I + II	11/34	24/56	6/40	1.32 (0.57-3.04); 0.648	0.46 (0.15-1.38); 0.260	0.96 (0.43-2.13); 1.000
III + IV	33/34	42/56	14/40	0.77 (0.41-1.44); 0.514	**0.36 (0.16-0.78); 0.015**	0.60 (0.33-1.07); 0.115
III	16/34	25/56	7/40	0.94 (0.44-2.02); 1.000	0.37 (0.13-1.00); 0.081	0.70 (0.34-1.44); 0.448
IV	17/34	17/56	7/40	0.60 (0.27-1.34); 0.303	0.35 (0.12-0.94); 0.059	0.50 (0.23-1.04); 0.094
Localization						
Colon	23/34	34/56	7/40	0.89 (0.45-1.77); 0.890	**0.25 (0.09-0.67); 0.008**	0.63 (0.33-1.20); 0.215
Rectum	21/34	32/56	12/40	0.92 (0.46-1.85); 0.967	0.48 (0.20-1.12); 0.138	0.74 (0.38-1.42); 0.464

P values were adjusted by the Bonferroni test (0.025). Bold text highlights statistically significant findings.
OR, odds ratio; CI, confidence interval.

lyzed; such a difference had not previously been established for any population. Furthermore, for the first time, individuals carrying the homozygous polymorphic genotype (A/A) of the rs10972727 variant showed a lower risk of developing CRC.

In this study, we also observed a significant association for sex and age (males and > 50 years) in patients carrying the A/A genotype, with OR values showing a protective effect in these individuals. On the other hand, a decreased risk of reaching advanced TNM stages (III + IV) was observed in individuals carrying the A/A genotype of the rs10972727 variant; however, such protective effect was not evident for stages III or IV separately. Furthermore, this same genotype was associated with tumor location in the colon.

In the haplotype analysis of these *RECK* variants (rs11788747-rs10972727), we found no haplotypes associated with CRC; however, Chen et al. (2014) found two haplotypes in the *RECK* gene, associated with lower risk in patients with non-small cell lung cancer, where the G risk allele of the rs11788747 variant is included.

As has been shown, the expression of *RECK* is crucial for angiogenesis and vasculogenesis, and its downregulation is implicated in tumor progression and metastasis (Clark et al. 2007; Noda and Takahashi 2007). Although in this study we did not analyze the expression of the *RECK* gene, it is feasible to suppose that the G and A polymorphic alleles, in the rs11788747 and rs10972727 variants respectively, could be increasing the expression of *RECK* and, consequently, reducing the risk of developing CRC. Furthermore, this same assumption could explain why these alleles are related to a protective role in reaching advanced stages of TNM in patients with CRC.

Further studies with larger sample sizes and from other populations are needed to validate the genetic effects of *RECK* polymorphisms in CRC. A limitation of this study was the absence of follow-up data and treatment response outcomes in these patients.

In conclusion, this is the first study revealing that the *RECK* rs11788747 and rs10972727 variants could be useful biomarkers as a protective factor for CRC. These polymorphic variants were also associated with advanced TNM stages and tumor localization.

Acknowledgments

This study was supported by Grants from “Fondo de Investigación en Salud. Instituto Mexicano del Seguro Social” (FIS/IMSS/PROT/G18/1822).

Conflict of Interest

The authors declare no conflict of interest.

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