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GSK3β Polymorphisms Are Associated with Tumor Site and TNM Stage in Colorectal Cancer

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Abstract

Background: Mutations and polymorphisms of the *GSK3β* gene have been associated with several diseases including Alzheimer disease, diabetes and cancer; however, to date, no variants of this gene have been associated with colorectal cancer (CRC). This study aims to explore, for the first time, the association of the *GSK3β* rs334558 and rs6438552 polymorphisms with CRC.

Methods: Genomic DNA from 330 CRC patients and healthy blood donors were analyzed. Identification of polymorphisms was made by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) methodology. Association was calculated by the odds ratio (OR) test.

Results: Patients carrying the C/T genotype for the rs334558 (T>C) polymorphism showed an increased risk for CRC (OR = 1.71, 95% CI: 1.05–2.79, *P* = 0.039); this association was also observed for TNM stage and tumor location. For the rs6438552 (T>C) polymorphism, the OR analysis showed that patients carrying C/T and C/C genotypes have a decreased risk for CRC (OR = 0.44, 95% CI: 0.27–0.70, *P* = 0.001 and OR = 0.24, 95% CI: 0.10–0.64, *P* = 0.001, respectively); this decreased risk was also evident in the stratified analysis by TNM stage and tumor location. Haplotype analysis of these 2 loci of *GSK3β* (rs334558 and rs6438552) showed differential distribution. The T-T and C-C haplotype was associated with a decreased risk of CRC, while the T-C haplotype was associated with an increased risk of CRC.

Conclusion: Our results denote that *GSK3β* gene polymorphisms play a significant role in promoting or preventing CRC. Additionally, variations in this gene are associated with the tumor site and the tumor-node-metastasis (TNM) stage in these patients.

Keywords: Colorectal cancer, *GSK3β* gene, *GSK3β* polymorphisms, *GSK3β* haplotypes

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Introduction

Colorectal cancer (CRC) is among the most frequent cancer types in American countries. In Mexico, the reported CRC incidence is 11.2 per 100 000 individuals.[1,2] Although little is known about CRC etiology, accumulated evidence support that it is mostly influenced by genetic factors, lifestyle and dietary factors.[3-5]

Glycogen synthase kinase 3 (GSK3) has been recognized as a multifunctional serine/threonine kinase involved in regulating many cellular functions such as proliferation, stem cell renewal, apoptosis, metabolism, differentiation, and cell motility. These multiple functions are related to the many substrates regulated by GSK3, including cell cycle components, transcription factors, and proteins involved in microtubule dynamics and cell adhesion. Alterations in GSK3 regulatory functions are associated with the onset and progression of diseases such as Alzheimer's, diabetes, and cancer.[6-9] Regarding *GSK3* and cancer, it is well accepted that *GSK3* is strongly implicated in tumorigenesis and cancer progression[10]; however, whether GSK3 acts as a tumor suppressor or tumor promoter seems to depend on the tumor type.[10] In some cases, silencing of its expression or suppression of its activity by phosphorylation of growth factor-stimulated kinases, such as Akt, has been associated with cancer progression.[9,11-13] GSK3 negatively regulates many proto-oncogenic proteins and cell cycle regulators through the Wnt/β-catenin and Hedgehog signaling pathways; however, GSK3 has also been associated with tumor progression by stabilizing β-catenin complex components in the Wnt signaling pathway and consequently displaying oncogenic properties. The Wnt/β-catenin signaling pathway is

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essential for the regulation of many cellular events such as cell proliferation, morphology, adhesion, migration, and structural remodeling.[14] In the Wnt signaling pathway, GSK3 phosphorylates defined β-catenin residues in the N-terminus, thus targeting the protein for ubiquitin/proteasome-mediated degradation.[15,16] Initiation of Wnt signaling through binding of the Wnt ligand to their receptors gives rise to inhibition of the Axin-APC-GSK3 complex. Subsequently, β-catenin becomes stabilized and translocates into the nucleus, where it acts on transcription factors of the TCF/LEF family, stimulating transcription of target genes such as *c-Myc, PPARD,* and *CCND1*.[17-19]

Although the mechanisms related to the intracellular localization of GSK3 are not fully elucidated, *GSK3β* isoforms are mainly located in the cytoplasm.[9,20] GSK3*β* is a serine/threonine kinase evolutionarily conserved and constitutively active. It is widely expressed in all tissues, with unusually high levels in the brain.[21] *GSK3β* has been described to have a dynamic nuclear location in response to cell cycle progression,[22] apoptotic stimuli,[9,23,24] and by interacting with the GSK-3-FRAT binding protein.[24] Nuclear GSK3*β* also plays a role in controlling the nuclear/cytoplasmic distribution of proteins such as *cyclin D1*, *STAT*, *GATA-4*, *c-Myc*, *NRF2*, *ZO-2*, *Snail*, and *p53*.[9,25,26] These studies support the participation of *GSK3β* in cancer processes; nevertheless, the specific role of GSK3*β* in cancer development has not been entirely understood.[9]

Single nucleotide polymorphisms of the *GSK3β* gene have been studied in several diseases such as bipolar disorders,[25,27-30] Alzheimer disease, Parkinson disease and different types of cancer,[31-35] Parkinson's[36,37] and different types of cancer.[38–44] Aristizabal-Pachon and Castillo, in 2017, reported for the first time an association between the SNP rs334558 (-50 T>C) of *GSK3β* gene with breast cancer. The rs334558 SNP is located in the promoter region (nt -171 to +29) of *GSK3β* gene and it is related with *in vitro* transcriptional strength; the T allele has a stronger activity. It has also been demonstrated that SNPs rs334558 and rs6438552 (intron 5) alter the *GSK3β* transcription and splicing and are associated with Parkinson's and other diseases likely related to *β* -catenin destruction complex alterations.[39] Although these polymorphisms have been strongly associated with breast cancer and other diseases, they have not been investigated in CRC development.

To the best of our knowledge, this is the first report examining allele and genotype distribution of these 2 *GSK3β* gene polymorphisms (rs334558 and rs6438552), assessing their possible association with CRC.

Materials and Methods

Subjects

The study included 330 individuals, 180 patients diagnosed with colorectal adenocarcinoma according to the clinicopathological criteria of the Specialty Hospital of the West National Medical Center of the Social Security Mexican Institute (IMSS) in Guadalajara, Jalisco, between 2014 and 2016. CRC was diagnosed according to the tumor-node-metastasis (TNM) classification. The control group included 150 nonrelated healthy volunteers, which were not age-matched with the patient group. A standard epidemiological questionnaire allowed us to collect personal data, including age, gender, drinking and smoking status, and familial history. Individuals with a previous history of cancer were excluded from the control group, while those who had undergone chemotherapy or radiotherapy were excluded from the patient group. Information about clinical and pathological features of the patients was obtained from the hospital records. All participants signed an informed consent form.

Genotyping

Genomic DNA was isolated from peripheral blood using standard methods.[45] The rs334558 (-50 T>C) and rs6438552 (157 T>C) polymorphisms in the *GSK3β* gene were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using the following primer pairs: rs334558-F: 5'- CTC GCT TCC TTC CTT CTT TT-3', rs334558-R: 5′-GAT TCC CAG ACG CCT GTT AC-3′ and rs6438552-F: 5'- CAG CTG CTT TGC ACT AAC AGA-3', rs6438552-R: 5′-TTA GGT GAC AAA CGC TTT CTT T-3′.[46] For the rs334558 polymorphism, PCR was performed using a 10 µL volume containing 100 ng DNA, 10X buffer (500 mM KCl, 100 mM Tris- HCl, and 0.1% Triton TMX-100), 2.0 mM $MgCl_2$, 200 µM dNTPs, 1 µM of each primer, and 2 U Taq DNA Polymerase. Denaturation was carried out at 95°C for 5 min, annealing at 54°C, and elongation at 72°C for 2 minutes and a final extension for 3 min at 72°C for 35 cycles. Five microliters of PCR products were digested with 3U *AluI* restriction enzyme (New England Biolabs, USA) at 37°C overnight, according to the manufacturer's instructions and separated on 6% polyacrylamide gels. Fragments observed by electrophoresis corresponded to 150 and 85 bp for the wild-type allele (T) and 235 bp for the polymorphic allele (C). The rs6438552 polymorphism was identified under the same PCR conditions except for the annealing temperature which was 56°C. Four units of Hpy188I enzyme restriction (New England Biolabs, USA) was used to digest 5 µL PCR products at 37°C overnight, according to the manufacturer's instruction. The digested products were separated on 6% polyacrylamide gels. Fragments observed by electrophoresis corresponded to 248 bp for the wild-type allele (T), and 180 bp and 68 bp for the polymorphic allele (C). The quality control for these assays was assessed by randomly selecting several samples that were re-genotyped by an independent technician. The observed concordance between genotyping assays was 100%.

Haplotype Analysis

We examined the haplotype frequencies in CRC patients and compared with those of controls. Also, the interaction of both *GSK3β* gene polymorphisms (rs334558 and rs6438552) by analyzing the combined effects of genotypes and haplotypes were assessed. Linkage disequilibrium and the haplotype frequencies were calculated using the Haploview 4.2 software.

Statistical Analysis

Allele and genotype frequencies were estimated by direct counting in both groups. Hardy-Weinberg equilibrium (HWE) was assessed by the Chi-square test. Differences in allele and genotype distributions and the clinical characteristics of patients and controls were also evaluated by chi-square test. Statistical analysis included Yates corrected chi-square test and odds ratio (OR) analysis. To establish the association of alleles or genotypes with CRC and the stratified TNM stage, OR and confidence intervals (CI) were calculated by the SPSS v17.0 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.

Table 1. Characteristics of Patients and Controls in This Study

	CRC Group n = 180	Control Group n = 150	*P* Value
Mean age (y), No. (%)	59.06 (13.48)	39.52 (12.28)	<0.001
Gender			
Female	72 (40)	58 (38.6)	0.805
Male	108 (60)	92 (61.4)	
Drinking status, No. (%)			
Ever	68 (37.7)	54 (36)	0.739
Never	112 (62.3)	96 (64)	
Smoking status, No. (%)			
Smokers	55 (30.5)	45 (30)	0.912
Non-smokers	125 (69.5)	105 (70)	
Family history of cancer, No. (%)			
Yes	35 (19.4)	-	
No	145 (80.6)	-	
Tumor localization, No. (%)			
Colon	120 (66.6)	-	
Rectum	60 (33.4)	-	
Clinical stage TNM, No. (%)			
I	1 (0.7)	-	
II	30 (16.6)	-	
III	62 (34.4)	-	
IV	87 (48.3)	-	

P values were calculated by chi-square test.

Results

Baseline Characteristics of the CRC Patients and the Control Group

Control group individuals (58 females and 92 males) and CRC patients (72 females and 108 males) were efficiently genotyped for rs334558 and rs6438552 polymorphisms in the *GSK3β* gene. Table 1 shows a comparative analysis of epidemiological and clinical data from CRC patients and control group. Significant differences, between both groups, were detected in age distribution ($P = 0.001$). In the CRC group, the average age was 59.06 years (range of 18 to 91 years); meanwhile, in the control group, the mean age was 39.52 years (range of 29 to 59 years). There were no significant differences between the control group and CRC patients concerning gender, smoking status, and drinking status.

Genotype Distribution and Allele Frequency of *GSK3β* Gene Variants

The comparative analysis of both variants in controls and CRC patients showed significant differences as measured by the OR (Table 2). In the control group, Hardy-Weinberg equilibrium was observed for the 2 analyzed SNPs (data not shown). The C/C genotype of the rs334558 polymorphism was found in 14.5% (26/180) of CRC patients and 10% (15/150) of the control group. The C/T genotype was observed in 60.5% (109/180) of CRC patients and 52.7% (79/150) of the control group, showing that patients with the C/T genotype also have an increased susceptibility for developing CRC (OR = 1.71; 95% CI = 1.05–2.79, $P = 0.039$). Allelic frequencies were significantly different, displaying that C allele carriers have increased susceptibility for developing CRC (OR = 1.41; 95% CI = 1.03–1.94, $P = 0.035$). Under a dominant pattern of allelic interaction, the C/C+C/T patients had an increased risk of CRC (OR = 1.78; 95% CI = 1.11–2.86, $P = 0.021$).

On the other hand, the C/C genotype of rs6438552 polymorphism was observed in 5% (9/180) of CRC patients and 12.7% (19/150) of control individuals, exhibiting a significant difference (OR= 0.24; 95% CI = 0.10–0.58, $P = 0.001$). The C/T genotype was observed in 33.8% (61/180) of CRC patients and 48.6% (73/150) of the control group. The risk analysis showed that C/T genotype patients have a decreased risk of developing CRC (OR = 0.44; 95% CI = 0.27–0.70, $P = 0.001$). Likewise, the allele frequencies were different (OR = 0.47; 95% CI = 0.33–0.67, $P = 0.001$); and patients with the C allele have a lower risk of developing CRC. Under a dominant pattern, the risk analysis showed that C/C+C/T individuals showed a lower risk of cancer than T/T individuals (OR = 0.40; 95% CI = 0.25–0.64, $P = 0.001$).

Association between *GSK3β* Gene Variants with TNM Stage and Tumor Location

Analysis of each SNP by TNM staging is presented in Table 3. Patients were stratified in 2 groups regarding the TNM status (stage III+IV *vs*. stage I+II). The risk analysis showed a significant difference only for the rs6438552

Table 2. Allele and Genotype Frequencies of the *GSK3β* Gene rs334558 and rs6438552 Polymorphisms in Patients and Controls

Genotype	Frequencies		OR (95% CI)	*P* Value
	CRC Group n = 180	Control Group n = 150		
GSK3β (rs334558)				
T/T	45 (25)	56 (37.3)	1.00 (Reference)	
C/T	109 (60.5)	79 (52.7)	1.71 (1.05–2.79)	0.039
C/C	26 (14.5)	15 (10)	2.15 (1.02–4.55)	0.064
C/C+ C/T *vs*. T/T	135 (75)	94 (62.7)	1.78 (1.11–2.86)	0.021
Allele				
T	199 (0.55)	191 (0.63)	1.00 (Reference)	
C	161 (0.45)	109 (0.37)	1.41 (1.03–1.94)	0.035
GSK3β (rs6438552)				
T/T	110 (61.2)	58 (38.7)	1.00 (Reference)	
C/T	61 (33.8)	73 (48.6)	0.44 (0.27, 0.70)	0.001
C/C	9 (5)	19 (12.7)	0.24 (0.10, 0.58)	0.001
C/C+ C/T vs. T/T	70 (38.8)	92 (61.3)	0.40 (0.25, 0.64)	0.001
Allele				
T	281 (0.78)	189 (0.67)	1.00 (Reference)	
C	79 (0.22)	111 (0.33)	0.47 (0.33, 0.67)	0.001

P values were calculated by chi-square test.

polymorphism in TNM stage III+IV patients, under the dominant model of inheritance C/C+C/T (OR = 0.38; 95% CI = 0.17–0.85, *P* = 0.027). Similar results were observed in the allele analysis: C allele showed an (OR = 0.47; 95% CI = 0.25–0.85, *P* = 0.020).

These TNM staging groups were compared with the control group, and significant differences were evident for both polymorphisms. In presence of the rs334558 C/T and C/C genotypes, risk analysis showed that these individuals had an increased susceptibility for developing CRC (OR = 1.75; 95% CI = 1.04–2.93, *P* = 0.044; and OR = 2.48; 95% CI = 1.15–5.37, *P* = 0.030, respectively). This susceptibility was also evident under the dominant model of inheritance (OR= 1.87; 95% CI = 1.13–3.08, *P* = 0.019). The allelic frequencies analysis showed increased risk to reach TNM stage III+IV (OR = 1.49; 95% CI = 1.07–2.06, *P* = 0.020) in individuals carrying the C allele.

Inversely, individuals carrying the C/T and C/C genotypes in the rs6438552 polymorphism showed a decreased risk for reaching TNM stages III+IV (OR = 0.37; 95% CI = 0.23–0.61, *P* = 0.001, and OR = 0.18; 95% CI = 0.07–0.50, *P* = 0.001 respectively). Likewise, we observe a decreased risk for developing CRC in C/T + C/C genotype individuals under a dominant model of inheritance (OR = 0.33; 95% CI = 0.21–0.54, *P* = 0.001). Analysis of allelic frequencies showed that patients carrying the C allele have a decreased risk to reach the TNM stage III+IV (OR = 0.41; 95% CI = 0.28–0.59, *P* = 0.001).

Analysis of each SNP by tumor location is shown in Table 4. Stratified analysis by tumor location showed

Table 3. Association between TNM Stage and the Genotypes Distribution for the rs334558 and rs6438552 Polymorphisms of the *GSK3β* Gene

SNP	Stage I+II CRC patients	Stage III+IV CRC patients	Stage III+IV vs. Stage I+II OR (95% CI)	*P* Value	Controls	Stage I+II CRC *vs*. Controls OR (95% CI)	*P* Value	Stage III+IV CRC *vs*. Controls OR (95% CI)	*P* Value
rs334558	n=31 (%)	n=149 (%)			n=150 (%)				
T/T	9 (29.1)	36 (24.1)	1.00 (Reference)		56 (37.3)	1.00 (Reference)		1.00 (Reference)	
C/T	20 (64.5)	89 (59.7)	1.11 (0.46–2.67)	0.990	79 (52.7)	1.57 (0.66–3.71)	0.404	1.75 (1.04–2.93)	0.044
C/C	2 (6.4)	24 (16.2)	3.00 (0.59–15.1)	0.298	15 (10)	0.82 (0.16–4.25)	1.000	2.48 (1.15–5.37)	0.030
C/C+CT	18 (58.1)	113 (75.8)	1.56 (0.64–3.79)	0.443	94 (62.7)	1.19 (0.50–2.83)	0.857	1.87 (1.13–3.08)	0.019
Allele									
T	38 (0.61)	161 (0.54)	1.00 (Reference)		191 (0.63)	1.00 (Reference)		1.00 (Reference)	
C	24 (0.39)	137 (0.46)	1.34 (0.77–2.35)	0.364	109 (0.37)	1.10 (0.63–1.94)	0.834	1.49 (1.07–2.06)	0.020
rs6438552	*n*=31 (%)	*n*=149 (%)			*n*=150 (%)				
T/T	13 (41.9)	97 (65.1)	1.00 (Reference)		58 (38.7)	1.00 (Reference)		1.00 (Reference)	
C/T	15 (48.4)	46 (30.8)	0.41 (1.18–0.93)	0.051	73 (48.6)	0.91 (0.40–2.07)	1.000	0.37 (0.23–0.61)	0.001
C/C	3 (9.7)	6 (4.1)	0.26 (0.05–1.20)	0.189	19 (12.7)	0.70 (0.18–2.73)	0.853	0.18 (0.07–0.50)	0.001
C/C+C/T	18 (58.1)	52 (34.8)	0.38 (0.17–0.85)	0.027	92 (61.3)	0.87 (0.39–1.91)	0.890	0.33 (0.21–0.54)	0.001
Allele									
T	41 (0.66)	240 (0.80)	1.00 (Reference)		189 (0.63)	1.00 (Reference)		1.00 (Reference)	
C	21 (0.34)	58 (0.20)	0.47 (0.25–0.85)	0.020	111 (0.37)	0.87 (0.49–1.55)	0.748	0.41 (0.28–0.59)	0.001

a significant difference for patients with colon tumor location and presence of the C/T (rs334558) and C/C (rs6438552) genotypes respectively. A significant difference under the dominant model of inherited was also detected for these polymorphisms. Similar results were observed in the analysis of allelic frequencies; individuals carrying the C allele of the rs334558 SNP showed an increased susceptibility for developing CRC with tumor location in the colon; meanwhile, the presence of C allele in the rs6438552 SNP showed a decreased risk of cancer in this location.

GSK3β Haplotypes

Four different haplotypes in the *GSK3β* gene were found (Table 5). Our results indicate that the 2 loci rs334558 and rs6438552 are in linkage disequilibrium. The most frequent haplotypes were T-T (CRC: 48%; controls: 61%), T-C (CRC: 30%; controls: 2%) and C-C (CRC: 15%; controls: 34%). We observed that the combination of the T allele in rs334558 and the T allele in rs6438552, as well as the C allele in rs334558 and the C allele in rs6438552 of the *GSK3β* gene, have a protective effect against cancer development. On the other hand, the combination of the T allele in rs334558 and the C allele in rs6438552 may be a factor for CRC susceptibility.

Discussion

A considerable number of studies about genetic mutations predisposing individuals to the development of CRC have been reported. The Wnt signaling is an essential pathway involved in morphogenesis, cell adhesion, differentiation, and proliferation. Constitutive activation or hyperactivity in the Wnt/β-catenin signaling has been associated with several types of cancers[47]; moreover, each Wnt/β-catenin pathway component has been strongly associated with different diseases. The GSK3β protein is a key controller of the Wnt pathway through the β-catenin destruction complex. The GSK3β protein also regulates different substrates and signaling pathways. Consequently, the mechanisms underlying the pro-tumor or anti-tumor actions of this molecule are intricate; however, it is accepted that the most significant effect on neoplastic transformation is likely mediated by its influence on the Wnt/β-catenin pathway.[48,49] The kinase activity of GSK3β is crucial for the β-catenin destruction complex; the β-catenin protein is phosphorylated by GSK3β and targeted for ubiquitin-mediated degradation to maintain a low level of cytoplasmic β-catenin. Activation of the Wnt pathway inhibits GSK3β, and consequently, β-catenin accumulates in the cytoplasm and translocates into the nucleus where it binds to the TCF and LEF transcription factors increasing their transcriptional activity.[50,51] Several TCF/LEF-targeted proto-oncogenes as *c-Myc* and *CCND1* and other genes involved in cell invasion/migration are drastically up-regulated.[52] Some studies have addressed the roles of GSK3β in human cancer reporting different effects

Table 4. Association between Tumor Location and the Genotypes Distribution for the rs334558 and rs6438552 Polymorphisms of the *GSK3β* Gene

SNP	Genotype	Controls n = 150 (%)	Colon Cancer n = 120 (%)	Rectal Cancer n = 60 (%)	Colon Cancer vs. Controls OR (95% CI)	*P* Value	Rectal Cancer vs. Controls OR (95% CI)	*P* Value
rs334558	T/T	56 (37.3)	21 (17.6)	24 (40)	1.00 (Reference)		1.00 (Reference)	
	C/T	79 (52.7)	80 (66.6)	29 (48.4)	2.70 (1.49–4.87)	0.001	0.85 (0.45–1.62)	0.756
	C/C	15 (10)	19 (15.8)	7 (11.6)	3.37 (1.45–7.84)	0.007	1.08 (0.39–3.00)	1.000
	C/C + C/T	94 (62.7)	99 (82.5)	36 (60)	2.80 (1.57–4.99)	0.001	0.89 (0.48–1.65)	0.839
	Allele							
	T	191 (0.63)	122 (0.50)	77 (0.64)	1.00 (Reference)		1.00 (Reference)	
	C	109 (0.37)	118 (0.50)	43 (0.35)	1.69 (1.19–2.39)	0.003	0.97 (0.62–1.52)	1.000
rs6438552	T/T	58 (61.2)	77 (64.1)	33 (55)	1.00 (Reference)		1.00 (Reference)	
	C/T	73 (33.8)	39 (32.5)	22 (36.6)	0.40 (0.24–0.67)	0.001	0.52 (0.27–1.00)	0.072
	C/C	19 (5)	4 (3.4)	5 (8.4)	0.15 (0.05–0.49)	0.001	0.46 (0.15–1.35)	0.235
	C/C + C/T	70 (38.8)	43 (35.8)	27 (45)	0.46 (0.27–0.77)	0.004	0.67 (0.36–1.25)	0.279
	Allele							
	T	189 (0.63)	193 (0.80)	88 (0.73)	1.00 (Reference)		1.00 (Reference)	
	C	111 (0.37)	47 (0.20)	32 (0.27)	0.41 (0.27–0.61)	0.001	0.61 (0.38–0.98)	0.056

Table 5. Association between the *GSK3β* Gene Haplotypes and Colorectal Cancer

Haplotype GSK3β rs334558-rs6438552		Frequencies		χ^2	OR (95% CI)	*P* Value
		CRC Group	Control Group			
T	T	86 (48%)	91 (61%)	10.63	0.59 (038–0.92)	0.019
C	C	27 (15%)	51 (34%)	33.92	0.34 (0.20–0.58)	0.001
T	C	54 (30%)	3 (2%)	89.04	21.0 (6.44–68.8)	0.001
C	T	13 (7%)	5 (3%)	5.95	2.25 (0.78–6.48)	0.130

on neoplastic cells.[53,54] Results in colon cancer suggest that GSK3β may also modulate the tumor development through changes in the Wnt/β-catenin pathway.[47,55]

The present study examines, for the first time, the potential association of the polymorphisms rs334558 (T>C) and rs6438552 (T>C) of the *GSK3β* gene with the development of CRC. Our results reveal that heterozygous rs334558 (C/T) patients show an increased CRC susceptibility. Likewise, it was evident that C/C and C/T genotype patients showed a significant association with TNM stages III+IV and with a colon location of the tumor. On the other hand, the statistical analysis revealed a decreased risk of cancer in individuals with C/T and C/C genotypes for the rs6438552 polymorphism, as well as a decreased risk in patients with TNM stage III+IV. These genotypes are preferentially found in patients with colon tumor location.

Several studies have demonstrated that both *GSK3β* gene polymorphisms (rs334558 and rs6438552) are significantly associated with neurological diseases such as bipolar disorders, schizophrenia, Parkinson and Alzheimer[21,56,57]; however, limited evidence is found about its role in cancer development. To date, there is only one report in which these variants have been associated with cancer. Particularly, Aristazabal-Pachon et al found by the first time an increased risk for breast cancer women with the presence the rs334558 C/T genotype; probably this polymorphism located in the *GSK3β* gene promoter region can alter the protein expression levels and generate some physiological consequence.[39] Kwok et al demonstrated that the T allele of the rs334558 polymorphism exhibits greater transcriptional activity (1.4 fold increased) than the C allele. Previously it has been shown that this allele T creates a new promoter binding site for an AP4 transcription factor, thus affecting his gene expression.[39,58]

Our results in patients with CRC are similar to those reported by Aristazabal-Pachon et al in breast cancer patients; in both studies exist a clear association of the C allele with cancer development, as well as with tumor progression.

According to the evidence presented by Lau et al, Russ et al, and Kwok et al,[39,44,58] the CRC patients should exhibit a diminished expression of *GSK3β* gene and, consequently, the neoplastic transformation in these patients would be related with reduced activity of GSK3*β* protein. In our opinion, the verification of such a hypothesis could be very interesting.[39,44,58]

On the other hand, Kwok et al demonstrated that rs6438552 T allele exhibited increased splicing (3.8 fold) compared to C allele, suggesting that the rs6438552 polymorphism modulates the utilization of spliced acceptor sites in downstream introns.[38] The mechanism for this splicing modulation is unknown, but these authors assumed that rs6438552 acts as a decoy splice site to recruit splicing factors that can affect the processing of adjacent exons. Such assumption suggests that the rs6438552 T/T genotype is a predictor of increased GSK3β activity due to improved splicing. This hypothetical mechanism could explain why, in our patients, the C allele is linked with a protective role for developing CRC. Although we did not analyze the *GSK3β* gene expression, it is probably altered in CRC patients, and perhaps this deregulation would depend on the location of the tumor (colon or rectum) or the TNM stage.

In this study, the *GSK3β* gene variants (rs334558 and rs6438552) seem to have opposite effects when analyzed individually; however, in the haplotype analysis, the combination of rs334558 T allele and rs6438552 C allele (T-C haplotype) significantly increases the CRC susceptibility (OR: 21.0; 95% CI: 6.44–68.8, $P = 0.001$).

Several reasons may have contributed to this paradoxical result. It is essential to remember that cancer is a complex disease resulting from interactions between environmental factors, genetic mutations, and epigenetic changes. The most plausible explanation for the controversial effect of these SNPs and the dual effect of GSK3β on cancer (pro-tumor or anti-tumor activity), is related with the decrease of its expression, caused by phosphorylation of the growth factor-stimulated kinases (anti-tumor action), and the negative regulation of proto-oncogenic proteins and cell cycle regulators through the Wnt/β-catenin pathway (cancer progression).[9,11-13]

In support to the results obtained in our study regarding the tumor location, studies achieved in Western countries found that two-thirds of CRC are located in the colon and only one third in the rectum.[59] Such a difference can be explained by the more considerable length of the colon and, consequently, a more extensive mucosa where a tumor could eventually start. Also, it is also clear that, since the biologic and histopathologic perspective, colon and rectum are distinct entities. Several genes, proteins, and miRNAs with regulatory functions have been identified related to colon cancer; meanwhile, a minimal amount of information is available for rectal cancer.[60]

These differences suggest that the colon is more susceptible than the rectum to develop cancer; however, in contrast to the absolute epidemiologic data, the relative carcinogenic risk of the rectal mucosa to develop cancer by far exceeds that of the colon mucosa in as much as the area at risk in the colon is definitely more extensive than the rectum. In other words, the rectal mucosa has at least four times higher risk for malignant transformation than the colon mucosa.[59] Regardless, the origin of the tumor (colon or rectum) should be seriously considered when selecting treatment strategies or stratifying patients for future clinical investigations.

In conclusion, this is the first study to explore the association of the *GSK3β* gene variants (rs334558 and rs6438552) with CRC. Our results suggest that the rs334558 polymorphism is a genetic risk factor for CRC,

while the rs6438552 polymorphism represents a protective element to CRC. Also, polymorphic variants in this gene are associated with tumor location and TNM stage in these patients. Although additional studies including larger samples and functional analysis of the polymorphisms are necessary to confirm and extend our findings, it is reasonable to propose that the *GSK3β* rs334558 and rs6438552 SNPs could be considered useful biomarkers to identify predisposition to or protection from CRC.

Authors' Contribution

MARR and PBN designed and supervised the research, interpreted the analysis and wrote the final manuscript. PZL, AMSS and TDPR performed the research, collected the data and analyzed the data. MPGA contributed to statistical analyses, outline preparation and drafting the manuscript. SEFM and JSC contributed to the discussion and critical revision of the manuscript.

Conflict of Interest Disclosures

The authors declare no conflict of interest.

Ethical Statement

This study was approved by the Ethical Committee 1305 (R-2014-1305-8) of West Biomedical Research Center, IMSS, and was conducted according to national and international ethical standards.

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