Interaction of CCND2, CDKN1A, *and* POLD3 *Variants in Mexican Patients with Colorectal Cancer*

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Cite this article as: Alvizo-Rodríguez CA, Flores-López BA, de la Luz Ayala-Madrigal M, et al. Interaction of *CCND2*, *CDKN1A*, and *POLD3* variants in Mexican patients with colorectal cancer. *Turk J Gastroenterol.* 2022;33(6):525-531.

ABSTRACT

Background: *Colorectal cancer is the second cause of death by cancer around the world. Sporadic colorectal cancer is the most frequent (75%), and it is produced by the interaction of environmental, epigenetic, and genetic factors. The accumulation of single-nucleotide variants in genes associated with cell proliferation, DNA repair, and/or apoptosis could confer a risk to cancer. The aim of this study was to analyze the gene–gene interactions among CCND2 (rs3217901), CDKN1A (rs1059234 and rs1801270), and POLD3 (rs3824999) variants in Mexican patients with colorectal cancer.*

Methods: *We collected peripheral blood samples from 185 patients with sporadic colorectal cancer before treatment and from 185 unrelated blood donors as the reference group; all participants signed an informed consent form. DNA extraction was performed by Miller and Cetyltrimethylammonium bromide (CTAB)/ Dodecyltrimethylammonium bromide (DTAB) methods. Polymerase chain reaction–restriction fragment length polymorphism followed by polyacrylamide gel electrophoresis stained with $AgNO_3$ methods were used to identify the variants rs3217901, rs1059234, rs1801270, and rs3824999. Odds ratio and single-nucleotide variant interaction were determined by single-locus analysis and Multifactorial Dimensionality Reduction software, respectively.*

Results: *No association was found for CCND2 and CDKN1A variants; yet, a significant association for the GG genotype, G allele, and recessive and additive models for the POLD3 variant was observed ($P < .05$). The single-nucleotide variant–single-nucleotide variant interaction revealed the combination rs1059234, rs3217901, and rs3824999 as the best model and the comparison showed an increased risk ($P < .05$).*

Conclusion: *Single-locus and gene–gene interaction analyses disclosed that both the rs3824999 (POLD3) variant and the combination of rs3217901 (CCND2), rs1059234 (CDKN1A), and rs3824999 (POLD3) genotypes increase the risk for colorectal cancer in Mexican population.*

Keywords: *Cell cycle, colorectal cancer, variants*

INTRODUCTION

Colorectal cancer (CRC) is one of the principal causes of death around the world and ranks third and second in incidence and mortality, respectively.[1] Colorectal cancer is usually a sporadic (75%) and multifactorial disease where genetics, epigenetics, and environmental factors are associated with an uncontrolled increased cell proliferation.[2,3] The adenoma–carcinoma sequence includes genetic changes that promotes the evolution of normal mucosa to invasive cancer. The accumulation and the interaction of these changes in colonic cells lead to CRC development.[3] Genome-wide association studies (GWAS) have related single-nucleotide variants (SNV) (mostly in non-coding regions) of cell cycle genes with CRC.[4] In this study, we selected 3 SNVs located in non-coding DNA of *CCND2*, *CDKN1A*, and *POLD3* genes and 1 exonic *CDKN1A* variant. *CCND2* is located on 12p13.32, contains 5 exons, and encodes for cyclin D2.[5] It is associated with CDK4 or CDK6 to regulate the G1 phase mainly through phosphorylation of RB protein.[6,7] The rs3217901

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Received: ***April 12, 2021*** *Accepted:* ***September 11, 2021*** *Available Online Date:* ***April 10, 2022***
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DOI: ***10.5152/tjg.2022.21223***

[NC_000012.12 (CCND2_v001):c.721-3673A>G] variant is located in intron 4.[8] *CDKN1A* gene is mapped on 6p21.2 and contains 4 exons.[5] This gene encodes for cyclin-dependent kinase inhibitor 1A also known as P21, a member of the CIP/KIP family, that inhibits cell cycle progression by binding to cyclin D/CDK4,6 and cyclin E/CDK2; this inhibitor is activated in response to DNA damage and oxidative stress.[9] Additionally, *CDKN1A* inhibits DNA synthesis by competing with the third subunit of DNA polymerase delta (POLD3) for the proliferating cell nuclear antigen (PCNA) binding.[10] In *CDKN1A* gene, the rs1801270 (NM_001374513.1:c.93C>T) is located in exon 3, changes the codon AGC to AGA, and leads to a replacement of serine by arginine (p.Ser31Arg) which in turn affects the α-helix protein structure implicated in the kinase inhibitory domain.[8,11] The rs1059234 (NM_000389.5:c.*20C>T) is located in the untranslated region 3′ (UTR-3′).[8] Finally, *POLD3* is located on 11q13.4, contains 12 exons, and encodes for a subunit of POLD3 involved in lagging-strand DNA replication and repair.[5] The rs3824999 [NC_00011.9 (POLD3_v001):c.1007-78T>G] variant is located in intron 9.[8] While the studies for rs1801270 and rs1059234 (*CDKN1A*) in CRC have not shown association, the results for rs3217901 (*CCND2*) and rs3824999 (*POLD3*) have been controversial.[12-19] The analysis of multiple DNA variants could determine which low penetrance genes are related to cancer in Mexican population, and the selection of variants in genes implied in cell cycle and the main process affected in cancer are the best choice. The aim of this study was to analyze gene–gene interactions among *CCND2*, *CDKN1A*, and *POLD3* variants in Mexican patients with CRC.

MATERIALS AND METHODS

Editorial Policies and Ethical Considerations

This study was approved by local ethics committee (register 6/2017-2018). Participants signed an informed consent for genetic studies in accordance with the Declaration of Helsinki, filled out a questionnaire regarding demographic data and risk factors to CRC, and finally agreed to a blood sample to identify the different SNV.

Main Points

- *The GG genotype of rs3824999 (POLD3) variant increased 2-fold risk for colorectal cancer in Mexican patients.*
- *The interaction of rs3217901 (CCND2), rs1059234 (CDKN1A), and rs3824999 (POLD3) variants increased 2-fold risk for colorectal cancer in Mexican patients.*
- *The rs3217901 (CCND2) and rs1059234 and rs1801270 (CDKN1A) variants are not associated with colorectal cancer in Mexican patients.*

Subjects

This study included 185 Mexican patients diagnosed by histopathological analysis with CRC and 185 healthy blood donors with no familial history of CRC; all patients were enrolled from "Dr. Juan I. Menchaca" Civil Hospital. Both groups were mestizos from Western Mexico.

Genotyping by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism

DNA extraction from blood samples was done by Miller et al[20] and DTAB/CTAB methods.[21] Once DNA concentration and purity was determined, each sample was subjected to PCR amplification, primers for *CCND2* and *POLD3* variants were designed with the Oligo Perfect Designer tool Invitrogen (Waltham, Massachusetts, USA) and New England Biolabs (Ipswich, Massachusetts, USA): forward 5′-TGGCTGGCAATGGTTAATTC-3′ and reverse 5′-AGACCTTGCTCCTCAACAAAATC-3′, and forward 5′-CTAAATCCCCTTTGCTGGACAT-3′ and reverse 5′-TTACCTTGACAGAAGGAGGTTCA-3′, respectively. The PCR conditions were 34 cycles of 94°C denaturation, 59°C annealing, and 72°C extension for both genes. The restriction enzymes were *Hin*1II (Invitrogen) and *Mlu*CI (New England Biolabs), respectively. Polymerase chain reaction products were digested at 37°C for 1 hour, with a final concentration of 1× buffer and 0.5 U/μL of restriction enzyme. The analysis of rs1059234 and rs1801270 variants of *CDKN1A* gene was done according to the method described by Cacina et al.[22] All the products were visualized in 6% polyacrylamide gel stained with $AgNO_3$. The size of PCR and restriction fragment length polymorphism (RFLP) products are described in Table 1.

Statistical Analysis

The allele and genotype frequencies were established by counting. Chi-squared test was used to determine the genotype differences, and Cochran–Armitage trend test under classical Mendelian inheritance patterns was done to evaluate the association of odds ratio (OR) with 95% CI. A $P < .05$ was considered as significant. Gene–gene interaction among the 4 SNVs was estimated in Multifactorial Dimensionality Reduction (MDR) software (version 3.0.2).[23] The best combination of the SNV was selected with Tuned ReliefF filter algorithm, and the search method configuration was exhaustive.

RESULTS

Demographic characteristics of both groups are shown in Table 2. In addition to the Hardy–Weinberg equilibrium observed for all analyzed SNVs, we found no association

Table 1. Size of PCR and Restriction Fragment Length Polymorphism Products of the Variants of *CCND2, POLD3,* and *CDKN1A* Genes

			Genotypes		
Gene/Variant	PCR (bp)	Enzyme	Homozygous (bp)	Heterozygous (bp)	Homozygous (bp)
CCND2/rs3217901	177	*Hin*1II	GG: 177	GA: 177, 148, and 29	AA: 148 and 29
POLD3/rs3824999	222	*Mlu*C1	GG: 222	GT: 222, 197, and 25	TT: 197 and 25
CDKN1A/rs1801270	272	*Blp*I	CC: 186 and 86	CA: 272, 186, and 86	AA: 272
CDKN1A/rs1059234	297	*Pst*I	CC: 258 and 39	CT: 297, 258, and 39	TT: 297

bp, base pair; PCR, polymerase chain reaction.

($P > .05$) with CRC for *CCND2* (rs3217901) and *CDKN1A* (rs1059234 and rs1801270) variants; yet, the G allele, GG genotype, and recessive and additive models of the *POLD3* polymorphism (rs3824999) exhibited a significant association (Table 3). Moreover, the correlation analysis between genotypes and clinical–pathological characteristics did not show any significant difference.

Table 2. Demographic Data of Colorectal Cancer Patients and Reference Group

Variables	Reference group, N	CRC Patients, N
Gender		
Female	73	76
Male	112	109
Physical activity		
No	78	81
Yes	107	86
ND		18
Diabetes mellitus type 2		
No	171	142
Yes	14	35
ND		8
Smoking		
No	93	84
Yes	92	94
ND		7
Alcohol		
No	53	91
Yes	132	84
ND		10
Age		
Mean	37	58.4
<60	172	93
>60	13	86
ND		6

ND, no data; CRC, colorectal cancer.

The gene–gene interaction analysis singled out the combination of rs1059234 (*CDKN1A*), rs3217901 (*CCND2*), and rs3824999 (*POLD3*) as the best model with a testing accuracy of 0.51 and a cross-validation consistency of 9/10 (Table 4, Figure 1A). The comparison between high- and low-risk genotype combinations had an OR of 2.33 and CI of 1.51-3.60, $P < .0001$. The synergic SNV–SNV interaction was between rs3217901 (*CCND2*) and rs1059234 (*CDKN1A*) (Figure 1B).

DISCUSSION

GWAS studies have evidenced a strong association of cancer development with non-coding (mainly intronic) variants. Although most of these variants have unknown functions, it is believed that some of them modulate alternative splicing, gene expression, mRNA transport or chromatin assembly, and nonsense-mediated decay, among other processes.[24]

Our findings that rs3217901 (*CCND2*) and rs1059234 and rs1801270 (*CDKN1A*) variants did not show significant association with CRC contrast with the opposite data reported for these variants in different types of cancer in several populations.[25-28] Regarding rs3217901 (*CCND2*), an association has been reported with oral cancer, but not with breast and esophageal cancer,[27,29,30] and contradictory results have been reported for ovarian and CRC.[14,19,25,31] Abulí et al[14] (2016) analyzed 1351 Spanish patients and did not find any relationship of this variant and CRC[14]; however, an association only for colon cancer but not for rectal cancer was reported by Jung et al[19] in Korean patients. The association studies for rs1059234 and rs1801270 (*CDKN1A*) also have opposite results; no association for both variants has been reported in several types of cancer including CRC.[12,32-34] However, for rs1059234, an association of risk with

Table 3. Genotypes and Allele Frequencies of *CCND2, CDKN1A,* and *POLD3* SNV in Patients with CRC and Reference Group

		CRC patients		Reference group			
Gene SNV	Genotype	n	%	n	%	*P*	OR (95% CI)
CCND2 rs3217901	GG	71	38.4	59	31.9		1.0 (Reference)
	GA	88	47.6	97	52.4	.218	0.754 (0418-1.182)
	AA	26	14.0	29	15.7	.360	0.745 (0.396-1.402)
	G	230	62.2	215	58.1		1.0 (Reference)
	A	140	37.8	155	41.9	.260	0.844 (0.629-1.134)
	Dominant model	71/114		59/126		.191	0.752 (0.490-1.154)
	Recessive model	159/26		156/29		.661	1.137 (0.641-2.017)
	CA trend under additive model					.249	0.848 (0.629-1.134)
CDKN1A rs1059234	CC	98	53	84	45.4		1.0 (Reference)
	CT	68	37	84	45.4	.097	0.694 (0.450-1.069)
	TT	19	10	17	9.2	.906	0.958 (0.468-1.961)
	C	264	71.4	252	68.1		1.0 (Reference)
	T	106	28.6	118	31.9	.336	0.857 (0.626-1.174)
	Dominant model	98/87		84/91		.145	0.738 (0.491-1.111)
	Recessive model	166/19		168/17		.725	0.884 (0.444-1.760)
	CA trend under additive model					.343	0.904(0.626-1.174)
CDKN1A rs1801270	CC	96	51.9	82	44.3		1.0 (Reference)
	CA	69	37.3	86	46.5	.086	0.685 (0.445-1.056)
	AA	20	10.8	17	9.2	.989	1.005 (0.494-2.025)
	C	261	70.5	250	67.6		1.0 (Reference)
	A	109	29.5	120	32.4	.381	0.870 (0.637-1.189)
	Dominant model	96/89		82/103		.145	0.738 (0.490-1.111)
	Recessive model	165/20		168/17		.603	0.835 (0.422-1.650)
	CA trend under additive model					.386	0.920 (0.637-1.189)
POLD3 rs3824999	TT	67	36	82	45		1.0 (Reference)
	TG	83	45	83	43.5	.371	1.224 (0.785-1.907)
	GG	35	19	20	11.5	.018*	2.142 (1.133-4.050)
	T	217	59	247	67		1.0 (Reference)
	G	153	41	123	33	.022*	1.416 (1.050-1.910)
	Dominant model	67/118		82/103		.111	1.402 (0.924-2.128)
	Recessive model	150/35		165/20		.028*	0.519 (0.287-0.939)
	CA trend under additive model					.025*	1.431 (1.050-1.910)

*$P < .05$.
CA, Cochran–Armitage trend test; CRC, colorectal cancer; SNV, single-nucleotide variant; OR, odds ratio.

Table 4. Models of Multifactor Dimensionality Reduction Analysis

Model	TA	CVC	OR (95% CI)	*P*
rs1801270	0.49	4/10	1.46 (0.96-2.21)	NS
rs1059234, rs3217901	0.50	5/10	1.86 (1.20-2.89)	.005
rs1059234, rs3217901, rs3824999	0.51	9/10	2.33 (1.51-3.60)	.0001

TA, testing accuracy; CVC, cross-validation consistency; NS, not significant; OR, odds ratio.

retinoblastoma, squamous cell carcinoma of the head and neck, and breast cancer has been reported, except for cervical cancer for which a protective role was established by Vargas-Torres et al.[28,29,35,36] An increased risk for rs1801270 has been concluded in head and neck cancer and acute lymphocytic leukemia, while it demonstrated a protective role in a meta-analysis for cervical cancer.[37-39] In our study, the only variant associated with CRC was rs3824999 (*POLD3*) with an increased risk similar to those reported in other studies.[14,16-18] However, there are also controversial results for CRC as no association was inferred in Spanish and Chinese population.[13,15] Moreover, no association was reported for gastric and esophageal cancer.[15,30] The differences in the genetic structure of the population could be related to the contradictory results in these association studies.

According to Ensembl Database,[40] the rs3824999 (*POLD3*) intronic variant is located in a regulatory region. The introns are removed during the mRNA processing;

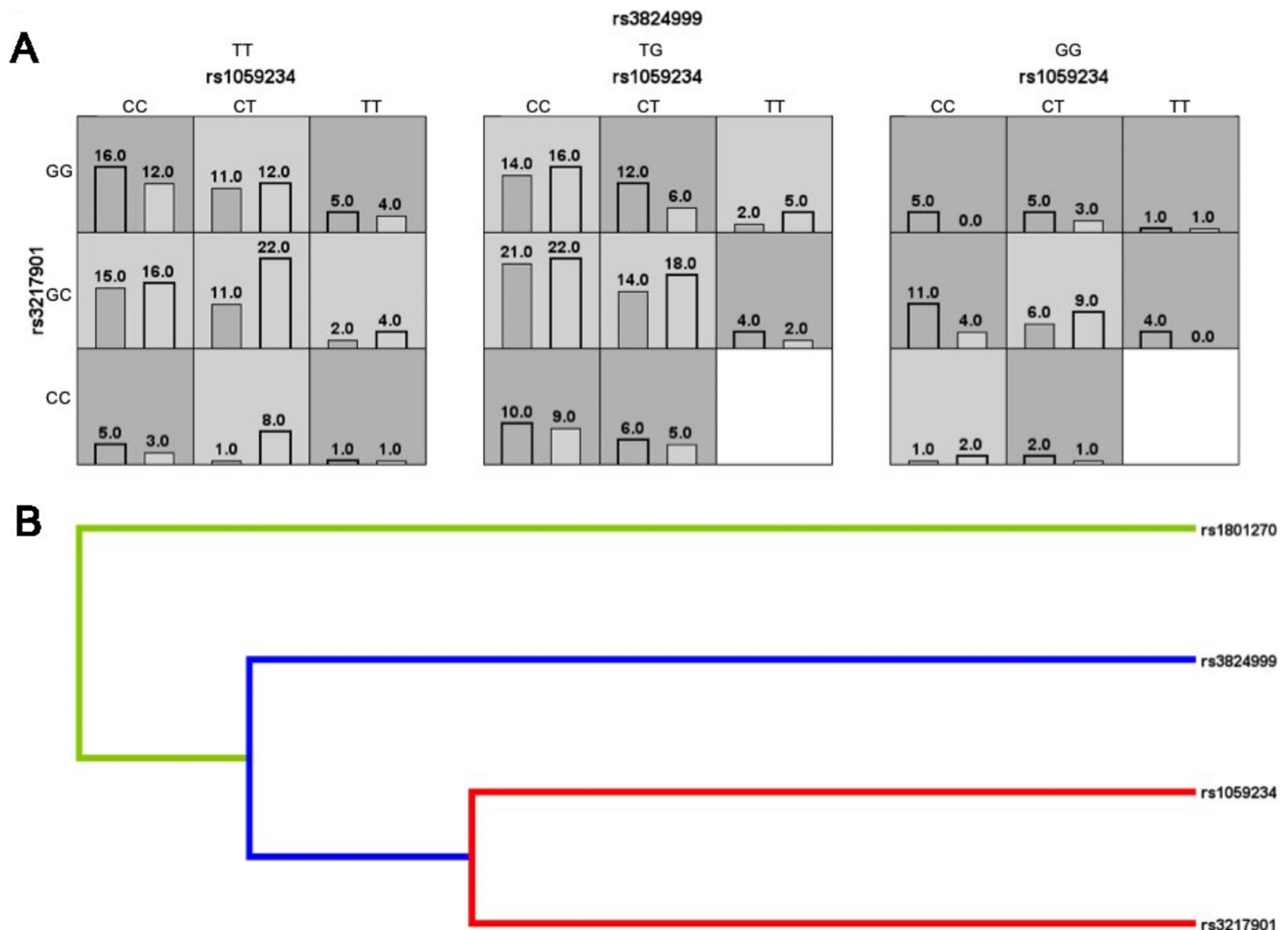

Figure 1. Multifactorial Dimensionality Reduction analysis. (A) Statistical model for 3-locus analysis. CRC cases (left bar) and controls (right bar). Dark and light gray bars denote "high risk" and "low risk" for CRC, respectively. Blank cells represent genotype combinations not observed in the analysis. (B) Dendogram shows the SNV–SNV interaction. Red line indicates synergistic interaction between rs3217901 (*CCND2*) and rs1059234 (*CDKN1A*), while green and blue lines represent redundancy. CRC, colorectal cancer; SNV, single-nucleotide variant.

however, in addition to the non-canonical and canonical splice sites, the introns could harbor genes for non-coding RNA and regulatory regions or enhancers for transcription factors,[41,42] therefore, the nucleotide change in this variant could affect gene expression and promote the cancer development.

The positive association found in different studies could reflect the usual pattern of cancer development resulting from the accumulation and interactions of distinct SNV that promote tumor progression and ultimately lead to an increased risk in certain populations.[43]

The gene–gene interaction analysis disclosed a 2-fold increased risk for the rs1059234 (*CDKN1A*), rs3217901 (*CCND2*), and rs3824999 (*POLD3*) genotype combination with the strongest synergy between the former 2 variants. This finding is not only consistent with the respective function (inhibition or induction) of these genes in cell cycle[6,7,9] but also evidences the complexity of cancer as a multifactorial disease with several genetic underlying factors and certain genotype combinations modifying the individual risk. Thus, among approximately 2000 genes listed in the Cancer Genetics Web (http://cancer-genetics.org/),[44] variants and interactions of almost 500 of them may modulate the risk for CRC development. Actually, the very small number of genes and SNVs analyzed along with the reduced sample size are the main limitations of the present study.

In conclusion, single-locus and gene–gene interaction analyses disclosed that both the rs3824999 (*POLD3*) variant and the combination of rs3217901 (*CCND2*), rs1059234 (*CDKN1A*), and rs3824999 (*POLD3*) genotypes increase the risk for CRC in Mexican population. Cancer is a multistep process and several DNA changes in addition to environmental factors are required for its development. Identifying the risk alleles in the population could make it possible in the future to establish a risk score for CRC development in Mexican population.

Ethics Committee Approval: The study was approved by local ethics committee of CUAltos (No. 6/2017-2018).

Informed Consent: Written informed consent was obtained from the patients who agreed to take part in the study.

Peer-review: Externally peer-reviewed.

Acknowledgment: CRAR and BAFL are students of PhD program in Human Genetics and were supported by CONACyT scholarship. We thank Dr. Horacio Rivera for the critical review of the manuscript.

Declaration of Interest: The authors have no conflict of interest to declare.

Funding: The authors declared that this study was funded by Universidad de Guadalajara.

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