Positive Association between the Polymorphic Variant CCND1 A870G and Colorectal Cancer in Ecuadorian Mestizo Population

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Abstract: *Background*: Colorectal cancer (CRC) is the fourth most common cause of cancer death worldwide and has an annual incidence of 917,000 cases. In Ecuador the CRC is the fifteenth most common form of cancer and the fourth leading cause of cancer deaths.

Aim: Our goal was to establish frequencies related to the polymorphic variants: (CA)n in the EGFR gene and A870G in the CCND1 gene and their influence on the development of CRC in the Ecuadorian population.

Methods: This is a retrospective case-control study consisting of colorectal cancer patients (n = 96 cancer tissues) compared to a control group (n = 62 adjacent healthy tissues). For the sequencing of the fragments, PCR and Sanger method was used.

Results: The polymorphic variant A870G in CCND1 has a genotype frequency for the common homozygous G/G = 0.69, for the heterozygous A/G = 0.25 and for the less frequent homozygous A/A = 0.06 in the control group. We studied 7 alleles, repeats 14-19 have been reported in other studies, but the 13 repeats allele was first described here. The most common number of repetitions was 18 with a frequency of 0.326 in patients and 0.25 in controls ($\chi 2 = 22.58$, $p < 0.01$). The odds ratio showed that the risk of developing colorectal cancer is 5 times greater if the individual is carrying the heterozygous G/A ($p < 0.01$). Meanwhile, if the individual is carrying the allele 'A' the risk is 4 times more likely to develop this disease ($p < 0.01$).

Keywords: Colorectal cancer, CCND1, A870G, polymorphic association.

INTRODUCTION

Colorectal cancer (CRC) is the third most commonly diagnosed cancer (1,366,744 cases) and the fourth cause of cancer-related death (697,087 cases) in 2012 [1-5]. Worldwide, the areas with a higher incidence of CRC per each 100,000 inhabitants are Oceania (34.8), Europe (29.5), and North America (26.12). In Ecuador, the CRC is the fifth most common form of cancer with an incidence of 10.7 and the seventh leading cause of cancer deaths (608,000 cases) in 2012 [6].

Most of CRC, regardless of its etiology, derives from adenomatous polyps that are distinguished by being visible bumps with significant molecular alterations [3, 4, 7-9]. A number of molecular markers for CRC have been recently identified, and some of them can influence the implementation of adjuvant therapies and the accurate prediction of the stage of the disease [10-12].

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Mutations and polymorphisms in the epidermal growth factor receptor (EGFR) are responsible for the gene over-expression and its relationship to a large number of solid tumors [11, 13, 14]. These alterations are associated with the disease progression, poor prognosis and chemotherapy resistance [11, 15, 16]. It has been observed that the length in the number of repetitions of the polymorphic variant (CA)n is inversely proportional to transcriptional activity of the gene. The number of repetitions must be found normally between 9 and 21 [11, 17]. Moreover, it has been demonstrated that genotypes with long repeats of (CA)n can be a predictor of the benefits of chemotherapy in CRC patients when compared to patients with a small number of repetitions [4, 11, 15, 16, 18].

Additionally, cyclins are responsible for cell cycle regulation [19-23]. Disrupting cyclins activity can cause imbalance in the cycle progression [24]. The CCND1 gene encodes the cyclin D1 protein that regulates the transition G1/S during the cell cycle [22, 23]. Protein overexpression occurs in more than one third of cases of colorectal cancer and adenomas. Common polymorphisms have a transition from G to A in codon

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242 of exon 4 in CCND1, showing splicing regulation of the transcript. There is evidence that CCND1 associates the A870G polymorphism with an increment (35%-50%) in the risk of developing adenomas and CRC [19, 21-23, 25].

Cancer stage at diagnosis determines treatment options. Approximately 39% of CRC are diagnosed when they are localized at (stage I/IIa), 26% after it has spread to regional lymph nodes (stage III) or directly beyond the primary site (stage IIb), 35% are diagnosed after the cancer has metastasized (distant stage ort stage IV). In Ecuadorian population with CRC the 5-year survival is 89.8% and for having distant metastases 12.9%. When mortality- incident radio is evaluated, we can look that for every 100 cases diagnoses there are 58 deaths [6]. In Ecuador, there are no studies related to molecular biomarkers for this disease. This research field is essential to determine how these polymorphic variants are presented in the Ecuadorian population in order to improve cancer prevention and treatment. The aim of this study was to determine whether there is an association between the risk of CRC and the genetic polymorphisms (CA)n in the EGFR gene or A870G in the CCND1 gene in patients from the Ecuadorian mestizo population.

MATERIALS AND METHODS

Participants and Samples

The Bioethics Committee of the Universidad de las Americas approved this retrospective case-control study following the Declaration of Helsinki. A total of 96 samples of CRC tissues, fixed in formalin and embedded in paraffin at different disease stages (II-IV) and, 62 adjacent healthy tissue samples came from the Solon Espinosa Ayala Oncological hospital and Pablo Arturo Suarez hospital in Quito, Ecuador. All participants signed their respective informed consent. Affected individuals were diagnosed with CRC during year 2013. Also data from clinical records of patients were accessed in order to complete the collection of pathological clinical and demographic variables; the average age of the participants was 62 ± 1.87 years, ranging from 24 to 83 years. This group had 76.09% of female individuals and 23.91% of male individuals. 55% of the colorectal cancer tissue samples had KRAS mutation.

DNA Extraction and Purification

The extraction and purification of DNA from patients and controls was performed using the PureLink Genomic DNA Kit (Invitrogen, Carlsbad, CA). DNA was extracted from seven to ten sections (6 µm) of formalin-fixed paraffin-embedded tissue samples previously cut with a microtome CUT 6062 (SLEE, Mainz, Germany). DNA concentrations were measured using a NanoDrop 2000 (ThermoScientific, Waltham, MA).

Genotyping

Genotyping was performed using the polymerase chain reaction (PCR) and DNA sequencing analysis. The PCR of the polymorphic variants (CA)n in the EGFR gene and A870G in the CCND1 gene produced fragments of 112 bp and 167 bp, respectively. A final volume of 15 µl (input 4 ng/µl of DNA) was used for each PCR reaction for EGFR and CCND1 genes. Each reaction consisted of 10.6 µl of Milli-Q water, 1.2 µl of DNA template (4 ng/ul), 10 mM of each deoxynucleotide triphosphate (dNTPs), 50 mM of $MgCl_2$, 2.5 U of Taq platinum DNA polymerase, 0.5 µl of 10x buffer (500 mM of KCl, 200 mM of Tris-HCl, pH=8.4), and 10 mM of forward (FW) and reverse (RV) primers detailed in Table **1** (Applied Biosystems, Austin, TX).

The amplification was carried out in a MultiGene thermocycler (Labnet Inc, Woodbridge, NJ). The reaction mixtures were incubated at 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 72 s and 1 cycle of 72 °C for 3 minutes. The resulting PCR fragments were revealed by electrophoresis in a 2% ultra-pure agarose gel stained with ethidium bromide. All gels were analyzed in an ImageQuant 300 transilluminator (General Electric,

Table 1: Sequence of Primers Used for the Amplification of the Region of interest of EGFR and CCND1 Genes

Genes	Polymorphic variants	Primer sequences	
EGFR	CA repetition	Forward	6FAM 5'TTTGAAGAATTTGAGCCAACC
		Reverse	TGCACACTTGGCACACTT
CCND1	A870G	Forward	GTGAAGTTCATTTCCAATCCGC
		Reverse	GGGACATCACCCTCACTTAC

Fairfield, SC). PCR amplified fragments were purified with Agencourt AMPure XP (Beckman Coulter, Miami, FL). Briefly, 10 µL of AMPure XP reagent and 10µL of PCR product were homogenized and incubated for 5 min at room temperature. Next, the tubes were placed in the magnetic plate for 10 min. The supernatant was removed and 200 µL of 70% ethanol was added. Subsequently, the tubes were incubated at 37 °C for 15 min to allow ethanol evaporation. Finally, 30 µL of 1X TE buffer (Tris EDTA pH 8) was added to the tubes homogenized by vortexing and 25 µL of the reaction were transferred to new tubes.

The genotypes were obtained through sequence analysis using the Genetic Analyzer 3130 (Applied Biosystems, Austin, TX). The PCR products were incubated at 96 °C for 3 min, followed by 25 cycles of 96 °C for 25 s, 50 °C for 5 s and 60 °C for 4 min in a MultiGene thermocycler (Labnet Inc, Woodbridge, NJ) using BigDye Terminator v3.1 sequencing standard (Applied Biosystems, Austin, TX). Once the product was amplified, it was purified using Agencourt Cleanseq (Beckman Coulter, Miami, FL). Finally, sequence analysis was performed using Sequence Analysis Software 5.3.1 (Applied Biosystems, Austin, TX), and the alignment was performed using Seq-Scape Software v2.6 (Applied Biosystems, Austin, TX).

Statistical Analysis

The information from the clinical records of the patients was collected and stored in a database. Allele frequencies, odds ratio (OR) and chi-square (χ^2) were calculated for the polymorphic variants. For the EGFR (CA)n variant, we proceeded to calculate the percentage of the population by the sum of the repeats of the two alleles as the initial diagnosis. All statistical analyses were performed using the IBM SPSS Statistics 22 software (SPSS Inc, Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

RESULTS

We have high yield of DNA isolated from FFPE samples. The concentration of the samples ranged from 6µg/µL to 320.2µg/µL. The amplified fragments were corroborated by horizontal electrophoresis in a 2% agarose gel. As shown in Figure **1**, the 112bp (A) and 167 bp (B) fragments coincide with the expected molecular weight determined by the weight marker used. After amplification and sequencing of the polymorphic variants, we determine samples with homozygous and heterozygous genotypes (Figure **2**).

According to the polymorphic variant (CA)n in the EGFR gene, the distribution of genotype frequencies is detailed in Table **2**. We found that the most frequent number of replicates was 18 with values of 0.250 for healthy tissue samples and 0.292 for samples with colorectal lesion. The less frequent repetition found was the number 19 with values of 0.047 in controls and 0.052 in patients.

The frequency obtained for controls and patients were (0.97) and (0.89) respectively. The sum of the CA dinucleotide repeats of the two alleles was lower than 35.

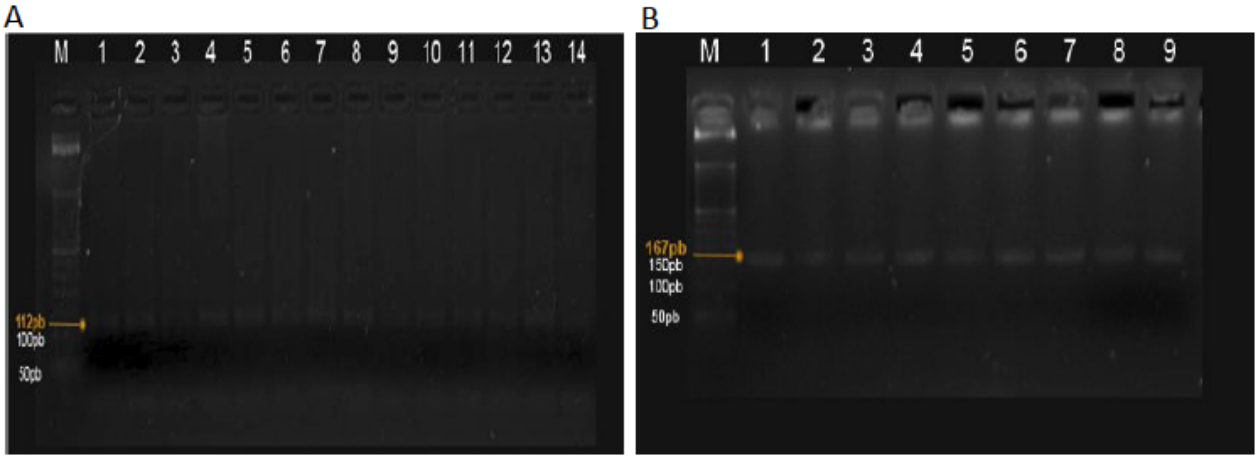

Figure 1: 2% agarose gel with the amplification results of the 112 and 167 bp fragments corresponding to the repetions of the gens EGFR and CCND1 respectively. (**A**) The proper amplification of the fragment of interest was substantiated, the 50bp molecular marker “M” is on the left of the gel. Wells 1 to 14 are some of the samples; all match the expected molecular weight, 112 bp belonging to the polymorphic variant CA in the EGFR gene. (**B**) From left to right, the well with the letter "M" belongs to the 50bp molecular weight marker used in electrophoretic run. The listed wells 1 to 9 show the presence of the 167 bp fragment belonging to the polymorphic variant A870G in CCND1.

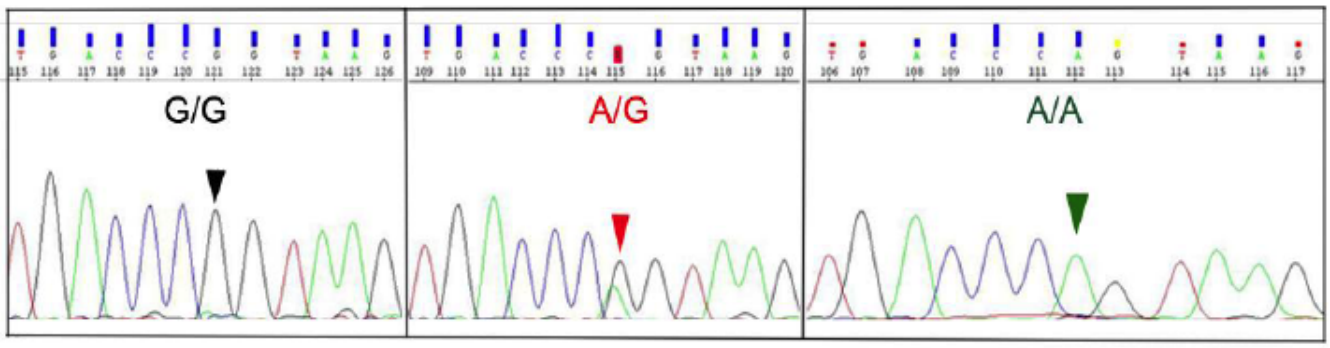

Figure 2: Sequencing electropherograms obtained from the CCND1 gene fragments. From left to right electropherograms of the homozygous G/G, the heterozygote A/G and the homozygous A/A can be observed.

Table 2: Frequencies for the Number of Dinucleotide Repeats CA in the EGFR Gene between Colorectal Cancer Samples and Healthy Tissue Controls

Polymorphism	N° repetitions	Healthy tissue	Frecuency HT*	Colorectal Lesion	Frecuency CL**
(CA)n in *EGFR*	REP 13	7	0,109	15	0,078
	REP 14	6	0,094	27	0,141
	REP 15	8	0,125	15	0,078
	REP 16	11	0,172	36	0,188
	REP 17	13	0,203	33	0,172
	REP 18	16	0,250	56	0,292
	REP 19	3	0,047	10	0,052

*HT: Healthy tissue.
**CL: Colorectal tissue.

The statistical analysis of the CA repetitions in the EGFR gene between patients and control samples showed a $\chi^2 = 1.962$ ($p > 0.05$) meaning that there are no significant differences between groups. According to these results we could determine that this polymorphic variant did not show significant difference between the variable and the diagnosis, as shown in Table **3**.

Genotype distribution and allele frequencies of the CCND1 A870G polymorphic variant are shown in Table **4**. Control samples had a genotype frequency for the common homozygous G/G=0.69, for the heterozygous A/G=0.25 and for the less frequent homozygous A/A=0.06. Allele frequencies were 0.81 for G and 0.19 for A. In patient samples the genotype frequencies for common homozygous G/G were 0.29, for the heterozygous A/G = 0.71 and for the less common homozygote A/A = 0.00. For the A and G alleles the frequency was 0.65 and 0.35 respectively.

We reported 7 alleles, from all of them, 14-19 repeats have been reported by other authors. However, the 13 repeats allele was first reported in this study. Moreover, the most common number of repetitions was 18 with a frequency of 0.326 in patients and 0.25 in controls. The chi-square for individuals in the healthy control group and in the colorectal cancer patient group was obtained ($\chi^2 = 22.58$, $p < 0.01$) indicating that there

Table 3: Number of Repetitions Frequencies of the Two Alleles of (CA)n in the EGFR Gene for Colorectal Lesions and Healthy Tissue Samples

Polymorphism	Σ Repetitions	Healthy tissue	Frecuency HT*	Colorectal Lesion	Frecuency CL**
(CA)n in *EGFR*	SSR (Σ ≤ 35)	31	0,97	85	0,89
	SLR (Σ > 35)	1	0,03	11	0,11

*HT: Healthy tissue.
**CL: Colorectal tissue.
SSR: Sum of Short Repeats.
SLR: Sum of Long Repeats.

Table 4: Analysis of Allelic and Genotypic Frequencies for the Polymorphism A870G in CCND1 Gene

SNP	Sample	Genotype	n	Genotypic frecuencies	Expected frequencies	Alelic frecuencies
A870G in *CCND1*	Controls	G/G	22	0,69	0,66	0,81
		A/G	8	0,25	0,30	
		A/A	2	0,06	0,04	0,19
		Total	32			
	Patients	G/G	25	0,29	0,42	0,65
		A/G	80	0,71	0,46	
		A/A	0	0,00	0,12	0,35
		Total	85			

are highly significant differences between the two groups.

Importantly, the homozygous A/A is not frequently found in the study population; in 127 samples only two had this genotype. The odds ratio was analyzed in order to determine which of the genotypes represent higher risk to develop colorectal cancer. We found that the risk of developing colorectal cancer is 5 times greater if the individual is carrying the heterozygous G/A. Meanwhile, if the individual is carrying the allele 'A' the risk is 4 times more likely to develop this disease, Table **5**.

Regarding the different tumor cell grade, clinical staging and the two polymorphic variants, we did not find any significance difference.

DISCUSSION

Colorectal cancer (CRC) is the fourth most common cause of cancer death worldwide; in 2012 the standardized incidence per 100 000 inhabitants affected with CCR was 1361 cases and 644 deaths, by the end of the year 2014 about 136 830 new cases of RCC and 50 310 deaths from this disease were reported [1, 26, 27].

In Ecuador the CR is the fifteenth most common form of cancer and the fourth leading cause of cancer death with 603 deaths by 2012. According to the latest report of the National Tumor Registry conducted between the years 2006-2010, an increasing incidence of CR was observed in the Ecuadorian population [6]. In Quito, the standardized incidence rate per 100 000 inhabitants has become 7.4 in men and 10.6 in women; with a total of 307 new cases of this cancer per year [6]. Worldwide, in the last 20 years, the mortality rate of CRC has declined. However, in Ecuador has been increasing, this may be due to the fact that other countries have opted for the preventive diagnosis of this disease and the removal of polyps before they become cancer [1, 3, 6-8, 26].

The majority of samples processed in Pathology laboratories are fixed in formalin. Although, this technique preserves the tissue structure, the extraction of nucleic acids, with optimum quality for molecular biology studies, is a significant problem. Formaldehyde causes crosslinking between nucleic acids and proteins. Moreover, it hydrolyzed phosphodiester bridges of the DNA [28, 29]. This could explain why not all the samples that we studied had high concentration of DNA. To improve the sample concentration, we conducted between 7 and 10 slides, depending on the size of the tissue from which we started the extraction of genomic DNA. Furthermore, we performed 2 xilol and ethanol washes, obtaining higher DNA concentrations from small samples (0.5 mm). These

Table 5: Analysis of the Odds Ratios of the Polymorphism A870G in CCND1

SNP	Genotype	Controls	Patient	Odds ratio	Confidence interval 95%	p value
A870G in CCND1	GG	22	25	Concerning		
	GA	8	50	5,5	2,147-14,092	p < 0.01**
	AA	2	0	0,468	0,345-0,635	p > 0.05^{NS}
	GA+AA	10	50	4,4	1,810-10,697	p < 0,01**

NS: not significant.
** Highly significant.

results were referenced earlier by Bustamante *et al*., 2011 [30].

The genetic variability among different populations worldwide is a resource used extensively in the study of cancer, as this condition is clearly involved with metabolism genes, repair pathways and cell cycle. Studies have shown that EGFR, a member of the ErbB receptor family is relevant in CRC, as the deregulation or expression of this gene occurs in 60-80% of cases [14]. The EGFR signaling pathway regulates cell differentiation, proliferation, migration, angiogenesis and apoptosis, all this processes are deregulated in cancer cells [14, 31]

The distribution of CA repeat in different populations worldwide has been reported as follows; in Caucasian and African-American population has been reported 8 alleles (14-21 repeats), the allele of 16 repeats with the most common allele frequencies of 0.43 and 0.42. 9 alleles in Chinese population (14-21 repeats and two rare alleles 9 and 22); the allele of 20 repeats was reported as the most common with a frequency of 0.65 [16]. In this study 7 alleles were reported, including the repeats 14-19 which have been reported in other populations. However, the 13 repeats allele, was reported for the first time in this study; and this shows the genetic variability of Ecuadorian population. Moreover, the most common number of repetitions was 18 with a frequency of 0.326 in CRC patients and 0.25 in healthy controls.

The length of the repeat (CA)n is inversely correlated with gene transcriptional activity. Studies *in vitro* and *in vivo* have demonstrated that the transcription of the gene declines when the number of CA dinucleotides increases, this establishes that a sum of the number of repetitions of both alleles (≤ 35) involves overexpression of EGFR, as well as being associated with poor clinical prognosis of the disease and resistance to chemotherapy and radiation in different solid carcinomas [15, 31]. Patients sum of repetitions of the variant (CA) n > 35 have the expectation of a better prognosis [11]. In the present study we found that a high percentage of the Ecuadorian population earned a sum of the number of repetitions ≤ 35, with frequencies of 0.89 in CRC patients and 0.97 in healthy controls.

Regarding cyclin D1 (CCND1), a Cashmere population study reported genotype frequencies in patients with colorectal lesion for the common homozygous G/G of 0.15; for the heterozygous A/G of 0.53 and for the less common homozygous 0.32. For healthy patients common homozygous G/G frequency was 0.26; for the heterozygous A/G was 0.47 and for the less frequent homozygous was 0.27 [21]. In Caucasian populations the frequency distribution of colorectal neoplasia samples for common homozygous G/G was 0.25; for the heterozygous A/G was 0.48 and for the less frequent homozygous was 0.26. in healthy patients the common homozygous G/G frequency was 0.25; for the heterozygous A/G was 0.51 and for the less frequent homozygous was 0.23 [5]. None of the above populations showed significant differences between patients and controls. In this study the homozygous G/G had a frequency rate of 0.66; the heterozygote of 0.30 and the homozygote A/A of 0.02, showing that in the case of healthy tissue samples we could observe higher prevalence of normal homozygous G/G; different to patient samples, where the heterozygote A/G is the most common.

Table 6: Frequencies of the A Allele of CCND1-G870A Polymorphism Associated with Increased Cancer Risk in Human Populations

Ethnicity	Country	Allele A frequency
Caucasian	FINLAND	0,77
	Australia	0,52
	Germany	0,53
	United states of America	0,59
	United kingdom	0,58
Asian	Singapour	0,39
	Taiwan	0,41
	India	0,41
	United states of America	0,51

Adapted from Pablan *et al*., 2008.

A recent study found that carriers of the A allele has increased risk of developing CRC, this has been observed in both models: co-dominance and dominance [25]. This is consistent with our research, since the A allele increases 4 times the risk of developing this disease. The same results were found in Caucasian populations but the opposite happens in populations of Asia and India; suggesting that ethnic differences play an important role along with genetic background and environmental factors in the development of this disease [21][32] Differences between ethnicities are shown in Table **6**.

It is clear that ethnic differences are important when evaluating genetic variability in a population. Several studies in Ecuador have shown that there are polymorphic variants widely studied in various parts of the world, but behave differently in the Ecuadorian population; for example the prostate cancer study of the V89L polymorphism in the SRD5A2 gene established that this polymorphic variant is a risk factor for the development of prostate carcinoma in Ecuadorians, different to studies in populations worldwide which reported a negative association between cancer and the polymorphism [33]. Our study corroborates that the frequencies found for the CA dinucleotide in EGFR gene were different from those found in other populations. From our preliminary work, we conclude that in colorectal lesions the most frequent genotype is A/G (0.71) and in healthy colon tissue is the homozygous G/G (0.69). According to our results, being an A allele carrier represents a 4 times greater risk of developing CRC. Therefore this biomarker could be used to assess risk of colorectal cancer among individuals.

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FINANCIAL DISCLOSURE

None.

CONFLICTS OF INTEREST

None.

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