

# APC Yin-Yang haplotype associated with colorectal cancer risk

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**Abstract.** The Yin-Yang haplotype is defined as two mismatched haplotypes (Yin and Yang) representing the majority of the existing haplotypes in a particular genomic region. The human adenomatous polyposis coli (*APC*) gene shows a Yin-Yang haplotype pattern accounting for 84% of all of the haplotypes existing in the Spanish population. Several association studies have been published regarding *APC* gene variants (SNPs and haplotypes) and colorectal cancer (CRC) risk. However, no studies concerning diplotype structure and CRC risk have been conducted. The aim of the present study was to investigate whether the *APC* Yin-Yang homozygote diplotype is over-represented in patients with sporadic CRC when compared to its distribution in controls, and its association with CRC risk. TaqMan<sup>®</sup> assays were used to genotype three tagSNPs selected across the *APC* Yin-Yang region. Frequencies of the *APC* Yin-Yang tagSNP alleles, haplotype and diplotype of 378 CRC cases and 642 controls were compared. Two Spanish CRC group samples were included [Hospital Clínico San Carlos in Madrid (HCSC) and Instituto Catalán de Oncología in Barcelona (ICO)]. Analysis of 157 consecutive CRC patients and 405 control subjects from HCSC showed a significant effect for the risk of CRC (OR=1.93; 95% CI 1.32-2.81; P=0.001). However, this effect was not confirmed in 221 CRC patients and 237 control subjects from ICO (OR=0.89; 95% CI 0.61-1.28; P=0.521). We found a significant association between the *APC* homozygote Yin-Yang diplotype and the risk of colorectal cancer in the HCSC samples. However, we did not observe this association in the ICO samples. These observations suggest that a study with a larger Spanish cohort is necessary to confirm the effects of the *APC* Yin-Yang diplotype on the risk of CRC.

## Introduction

Colorectal cancer (CRC) is one of the most common malignancies and the second leading cause of cancer-related death in Spain. An individual's life-time risk of developing CRC is 6%, with over 90% of cases occurring after the age of 50 years. Together, these facts indicate that CRC is an important health concern (1,2).

Population-based genetic association studies (case control studies) are the most widely used study designs with which to determine the impact of genetic variants on the risk of developing a particular complex disease (3,4).

In this way, hundreds of association studies have been performed in order to elucidate the genetic contribution in complex diseases, such as cancer. Through these studies several low penetrance genes have been found to behave as cancer risk modifiers, contributing to the understanding of tumor formation in many types of cancers and leading to advances in diagnosis and therapy.

Due to the high frequency of genetic polymorphisms in the human genome and the number of possible haplotypes in a particular chromosomal region, it is difficult to establish the level of similarity between different haplotypes (5). However, this problem was solved a few years ago through the identification of an exceptionally abundant Yin-Yang haplotype pattern extending 75-80% of the entire human genome (6). This peculiar pattern is defined as two mismatched haplotypes (Yin and Yang) representing the majority of the existing haplotypes in a particular genomic region. An increase of the recombination rate has been observed in human genome regions with Yin-Yang haplotype structure (6). The human adenomatous polyposis coli (*APC*) gene is localized on chromosome 5q21-227 and encodes a multifunctional protein that participates in several cellular processes: cell adhesion and migration, signal transduction, microtubule assembly and chromosome segregation (7). A somatic mutation in *APC* is the most common acquired genetic alteration in colorectal adenomas (CRA) and carcinomas (CRC) and one of the earliest mutation events in sporadic colon cancer (8-10). Somatic inactivation occurs in more than 80% of sporadic CRC and it follows the Knudson hypothesis, showing similar incidences of LOH and gene mutation as second events (11,12). These sporadic tumors, which do not present somatic inactivation

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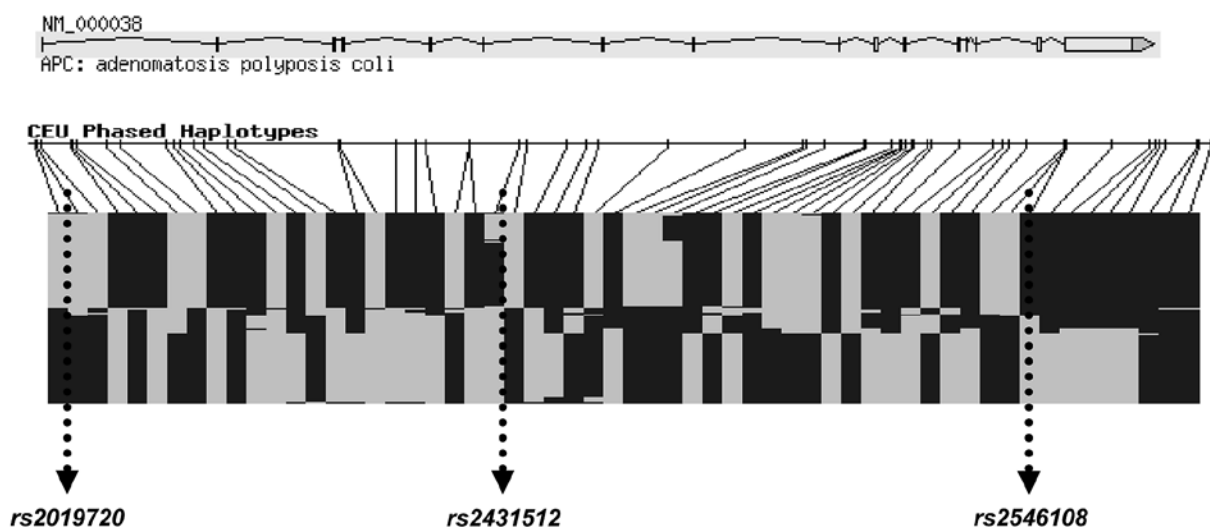


Figure 1. CEU phased haplotypes for *APC*. From the International HapMap Project website. Graphic representation of all related SNPs located in the *APC* gene sequence. Alternative alleles are indicated in black and grey. Black arrows show the three htSNPs selected across the Yin-Yang haplotype gene sequence.

of *APC* (less than 20%), show tumor microsatellite instability (MSI) (13) while its carcinogenic pathway is different, being affected by the mismatch repair pathway suppressor tumor genes (14).

The *APC* gene is our candidate gene, since it functions as a gatekeeper of colorectal neoplasia. *APC* shows a Yin-Yang haplotype pattern described in a European (CEU) population (5) and also in a Spanish population (15).

Several association studies have been published on *APC* gene variants and CRC risk. Many of them analyzed separate SNPs (16-20), while a few others analyzed the combination of various SNPs (haplotypes) (21,22). However, no studies have been published involving diplotype structure and colon cancer risk.

In this study, we searched for other genetic factors that act as risk modifiers apart from those affecting the DNA sequence. For instance, the similarity between homologous chromosomes at particular loci may affect the recombination rate in these loci and, therefore, may favor the inactivation of particular recessive genes. To test this hypothesis, we present an association study using a specific approach in which this particular structural characteristic (diplotype) acted as a genetic variant. The aim of the present study was to investigate whether the *APC* Yin-Yang homozygote diplotype is over-represented in patients with stable microsatellite (MSS) sporadic CRC when compared to its distribution in controls, and to establish whether this diplotype is a risk factor for CRC in a Spanish population.

## Patients and methods

**Study populations.** Cases and controls were recruited from two public hospitals in Spain; Hospital Clínico San Carlos in Madrid (HCSC samples) and Instituto Catalán de Oncología in Barcelona (ICO samples).

HCSC samples included 157 CRC cases with a mean age at diagnosis of 67 years (range 25-90) recruited between 1998 and 2005 at the Molecular Oncology Unit, and 405 controls with a mean age of 65.8 years (range 25-90) recruited

between 2001 and 2008 at the Clinical Pathology Laboratory. Specifically, eligible individuals had no personal or family history of cancer.

ICO samples included 221 CRC cases with a mean age at diagnosis of 70.6 years (range 31-91) recruited between 1990 and 2005 at the Catalán Institute of Oncology, and 237 controls with a mean age of 67.5 years (range 35-91) recruited between 1990 and 2001 at the National Blood Transfusion Centre.

Informed consent was obtained from all participants, and the study was approved by the Institutional Review Board of HCSC.

Cases and controls from HCSC and ICO were matched for age and gender.

**DNA isolation.** Genomic DNA was isolated from peripheral blood lymphocytes by a salting out procedure (23) or by automatic DNA extraction (Magnapure®; Roche) according to the manufacturer's protocol. Tumor DNA was isolated from the dissection of tumor cells from paraffin-embedded tissue (selected on the basis of an H&E-stained slide), using the QIAamp DNA FFPE Tissue kit extraction method. DNA was quantified using Nanodrop® R (ND 1000) and diluted to a final concentration of 50 ng/μl for genotyping.

**Microsatellite instability (MSI).** MSI analysis was performed in all paired tumor-normal tissue DNA samples testing the Bethesda (24) panel of five markers (D2S123, D5S346, D17S250, BAT 25 and BAT 26). PCR amplifications were performed with the HNPCC Microsatellite Instability kit (Roche Diagnostic, Basel, Switzerland) according to the supplier's instructions. Products were analyzed in an ABI310 genetic analyzer. Tumors were classified as MSS when none or just one of the five Bethesda markers showed instability.

**Genotyping.** The *APC* Yin-Yang haplotype was described by Ribas *et al* (15) in a Spanish population. We chose three tagged SNPs (tgSNPs) (rs2019720, rs2431512 and rs2546108) for the two frequent haplotypes. Although we needed only one tgSNP

Table I. Characteristics of the selected tgSNPs (NCBI).

NCBI SNP reference	rs2019720	rs2431512	rs2546108
Location on gene (chromosome 5)	Intron 1 (112.102.168)	Intron 6 (112.146.855)	Intron 11 (112.189.368)
Reference of SNP alleles	A/G	C/T	A/C
Ancestral allele (frequency in CEU)	A (0.508)	C (0.5)	C (0.492)
Dyes	VIC - allele A FAM - allele G	VIC - allele C FAM - allele T	VIC - allele A FAM - allele C
Heterozygosity $\pm$ standard error	0.494 $\pm$ 0.055	0.495 $\pm$ 0.047	0.495 $\pm$ 0.048
TaqMan assay	C_1120624_10	C_3162979_10	C_9389410_10
Yin haplotype	G	T	A
Yang haplotype	A	C	C

Table II. Haplotype frequencies in the case and control groups.

Population	No.	GTA (Yin) no. (%)	ACC (Yang) no. (%)	Others no. (%)
HCSC				
CRC	314	166 (52.9)	146 (46.5)	2 (0.6)
Control	810	418 (51.6)	379 (46.8)	13 (1.6)
ICO				
CRC	442	233 (52.7)	203 (45.9)	6 (1.4)
Control	474	242 (51.1)	226 (47.7)	6 (1.3)
All samples				
CRC	756	399 (52.8)	349 (46.2)	8 (1.1)
Control	1,284	660 (51.4)	605 (47.1)	19 (1.5)

HCSC, Hospital Clínico San Carlos in Madrid; ICO, Instituto Catalán de Oncología in Barcelona.

to test our hypothesis, we used three to confirm the Yin-Yang haplotype structure of our study population. The three tgSNPs were selected through the HapMap Phased Haplotype plot and distributed along the *APC* Yin-Yang region (Fig. 1). Their heterozygosity index was close to 0.5 (Table I). Genotyping was carried out by nuclease assay (TaqMan®). TaqMan genotyping reagents were designed by Applied Biosystems as Assay-by-Design (Table I). PCR reaction was carried out according to the manufacturer's recommended protocols in an ABI PRISM® 7900HT System (Applied Biosystems). The analysis was carried out with the SDS 2.2.1 ABI software.

**Statistical analysis.** Deviations from Hardy-Weinberg equilibrium were tested using the Chi-square test. Two-sided Chi-square tests were used to assess the differences in *APC* diplotype frequency distributions between cases and controls. Power calculations were performed by Epi-Info 6 program.

## Results

We performed a case-control study to investigate the possible association of the three tgSNPs selected across the *APC* gene (Fig. 1) with CRC risk. Genotyping of the rs2019720,

rs2431512 and rs2546108 was carried out in 157 cases and 405 controls from the Hospital Clínico San Carlos in Madrid, Spain (HCSC samples). All tgSNPs tested were in Hardy-Weinberg equilibrium ( $P=0.17$ ,  $0.19$  and  $0.17$ , respectively). The genotyping data are available upon request. Yin-Yang haplotypes were established in the HCSC samples. The frequencies in the CRC cases and controls were 52.25% for the GTA haplotype (Yin) and 46.65% for the ACC haplotype (Yang). The remaining 1.1% belonged to other haplotypes. The *APC* Yin-Yang alleles (GTA and ACC, respectively) comprised 98.8% of all of the haplotypes (Table II).

After genotyping, samples were divided into two subgroups; the homozygote diplotype (GTA/GTA or ACC/ACC) and the heterozygote diplotype (GTA/ACC). Rare haplotypes were not detected in homozygosis, so they were included in the heterozygote diplotype group (Table III). We observed an increase in the homozygote frequency in the HCSC cases when compared to the HCSC-controls (OR=1.93; 95% CI 1.32-2.81;  $P=0.001$ ). Statistical power was >80%.

We subsequently decided to validate the positive results in another Spanish group of cases and controls. We genotyped 221 cases and 237 controls from the Instituto Catalán de Oncología in Barcelona (cases and controls are described

Table III. Diplotype frequencies in the case and control groups.

Population	No.	Homo (%)	Hete (%)	OR	95% CI	P-value
HCSC						
CRC	157	96 (61.1)	61 (38.8)	1.93	1.32-2.81	0.001
Control	405	182 (44.9)	223 (55.1)			
ICO						
CRC	221	109 (49.3)	112 (50.7)	0.89	0.61-1.28	0.521
Control	237	124 (52.3)	113 (47.7)			
All samples						
CRC	378	205 (54.2)	173 (45.8)	1.30	1.01-1.68	0.043
Control	642	306 (47.7)	336 (52.3)			

Homo, homozygote diplotype (ACC/ACC and GTA/GTA); Hete, heterozygote diplotype (ACC/GTA and others); OR, odds ratio; CI, confidence interval. HCSC, Hospital Clínico San Carlos in Madrid; ICO, Instituto Catalán de Oncología in Barcelona.

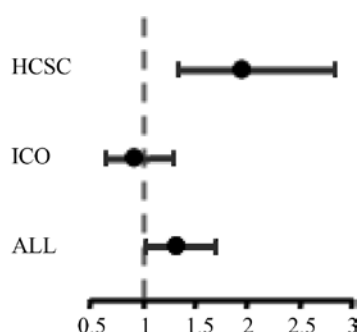


Figure 2. Odds ratio of APC homozygote diplotypes for HCSC and ICO study populations.

in Patients and methods). The SNPs were also in Hardy-Weinberg equilibrium ( $P=0.33$ ,  $0.23$  and  $0.37$ , respectively). The frequencies for the Yin-Yang haplotypes were 51.9 for GTA (Yin), 46.8 for ACC (Yang) and 1.3 for other haplotypes (Table II). We found very similar haplotype frequencies in the HCSC and ICO samples. In relation to the diplotype results we did not find any significant difference in the frequency of the homozygote diplotype between the cases and controls (OR=0.89; 95% CI 0.61-1.28;  $P=0.521$ ) (Table III).

When we combined the samples from HCSC and ICO to analyze both populations (378 cases and 642 controls) the homozygote diplotype frequency in the cases was 54.2% (Table III). A slightly significant result was noted (OR=1.3; 95% CI 1.01-1.68;  $P=0.043$ ) (Table III, Fig. 2).

## Discussion

With the aim of verifying our hypothesis that over-representation of the APC Yin-Yang homozygote diplotype occurs in MSS sporadic CRC populations, we genotyped three tgSNPs spanning the gene (Fig. 1, Table I).

We assessed the haplotype distribution in the cases and controls of the HCSC and ICO populations. All groups showed similar frequencies of Yin-Yang haplotypes (Table II), indicating no over-representation of a single haplotype in our study group.

Moreover, we found similar frequencies of APC Yin-Yang haplotypes (98.8%) as Ribas *et al* (25) in a Spanish population (84.1%).

We carried out a case-control analysis of the Yin-Yang diplotype in the HCSC population and observed a significantly increased frequency of the homozygote diplotype in the case group; 61.1 vs. 44.9% in the control group (OR=1.93; 95% CI 1.32-2.81;  $P=0.001$ ).

In order to replicate our positive results, we repeated the analysis in a second Spanish MSS sporadic CRC population confirmed by ICO and compared this group to a control group of the same origin. In this case, the ICO sample did not show a difference between the cases and controls; 49.3 vs. 52.3% (OR=0.89; 95% CI 0.61-1.28;  $P=0.521$ ).

In the HCSC samples, we found differences in the diplotype distribution between the cases and controls. In contrast, we did not find any difference in the ICO samples. Since the selection criteria for the cases were the same in both populations, these discrepancies may be attributed to the eligibility criteria for the controls. The ICO controls were enrolled at the National Blood Transfusion Centre, while the HCSC controls were enrolled at the Clinical Pathology Laboratory after filling out a questionnaire, and only those with no history of cancer were chosen. Selection criteria for the ICO control group did not exclude the enrollment of subjects affected with cancer. This fact may also explain the similar homozygote frequencies found in our study between the ICO cases and controls.

Environmental factors also affected the disease prevalence in a different manner in both the populations, so these may have contributed to the observed differences in the diplotype distribution in both samples.

Finally, notably, enough statistical power (>80%) was present to detect an OR as high as 1.9 in the HCSC sample, while enough power was not present to detect a low OR in the ICO samples. Therefore, we cannot discard a weaker effect of the Yin-Yang homozygote diplotype in the ICO population, which could not be detected with our sample size.

To resolve this problem, additional studies using different sets of patients and control subjects are warranted, since supporting evidence exists for an association between the APC



Yin-Yang homozygote diplotype and CRC risk in the HCSC population.

Although the association between the *APC* Yin-Yang homozygote diplotype and CRC risk remains to be confirmed, a singular strategy to test the hypothesis has been presented. In this study, a structural genetic characteristic (such as Yin-Yang diplotype) was compared in different groups instead of genetic variants and, therefore, other different mechanisms (such as allelic recombination or gene conversion) were tested instead of allelic functionality. Studies on SNPs included in the Yin-Yang structure and CRC risk may provide more information than isolated SNPs. The selection of cases and controls from different sources must be based on identical criteria in order to compare both populations. A more extensive study is required to support the evidence for the association between the *APC* Yin-Yang homozygote diplotype and CRC risk.

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### References

1. Fearon ER and Volgestein B: A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990.
2. De la Chapelle A: Genetic predisposition to colorectal cancer. *Nat Rev Cancer* 4: 769-779, 2004.
3. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L and Nickerson DA: Selecting a maximally informative set of single nucleotide polymorphisms for association analyses using linkage disequilibrium. *Am J Hum Genet* 74: 106-120, 2004.
4. Gabriel SB, Scaffner SF, Nguyen H, *et al*: The structure of haplotypes blocks in the human genome. *Science* 296: 2225-2229, 2002.
5. The International HapMap Consortium: A haplotype map of the human genome. *Nature* 437: 1299-1320, 2005.
6. Zhang J, Rowe WL, Clark AG and Buetow KH: Genomewide distribution of high-frequency, completely mismatching SNP haplotype pairs observed to be common across human populations. *Am J Hum Genet* 73: 1073-1081, 2003.
7. Bodmer WF, Bailey CJ, Bodmer J, *et al*: Localization of the gene for familial adenomatous polyposis on chromosome 5. *Nature* 328: 614-616, 1987.
8. Fodde R: The *APC* gene in colorectal cancer. *Eur J Cancer* 38: 867-871, 2002.
9. Senda T, Iizuka-Kogo A, Onouchi T and Shimomura A: Adenomatous polyposis coli (*APC*) plays multiple roles in the intestinal and colorectal epithelia. *Med Mol Morphol* 40: 68-81, 2007.
10. Powell SM, Zilz N, Beazer-Barclay Y, *et al*: *APC* mutations occur early during colorectal tumorigenesis. *Nature* 359: 235-237, 1992.
11. Rowan AJ, Lamlum H, Ilyas M, *et al*: *APC* mutations in sporadic colorectal tumors: a mutational 'hotspot' and interdependence of the 'two hits'. *Proc Natl Acad Sci USA* 97: 3352-3357, 2000.
12. Segditsas S, Sieber OM, Rowan A, *et al*: Promoter hypermethylation leads to decreased *APC* mRNA expression in familial polyposis and sporadic colorectal tumours, but does not substitute for truncating mutations. *Exp Mol Pathol* 85: 201-206, 2008.
13. Ionov Y, Peinado MA, Malkhosyan S, Shibata D and Percho M: Ubiquitous somatic mutations in simple repeated sequences reveal a new mechanism for colonic carcinogenesis. *Nature* 363: 558-561, 1993.
14. Markowitz SD and Bertagnolli MM: Molecular origins of cancer: molecular basis of colorectal cancer. *N Engl J Med* 361: 2449-2460, 2009.
15. Ribas G, Gonzalez-Neira A, Salas A, *et al*: Evaluating HapMap SNP data transferability in a large-scale genotyping project involving 175 cancer-associated genes. *Hum Genet* 118: 669-679, 2006.
16. Laken SJ, Petersen GM, Gruber SB, *et al*: Familial colorectal cancer in Ashkenazim due to a hypermutable tract in *APC*. *Nat Genet* 17: 79-83, 1997.
17. Gryfe R, Di Nicola N, Gallinger S and Redston M: Somatic instability of the *APC* I1307K allele in colorectal neoplasia. *Cancer Res* 58: 4040-4043, 1998.
18. Frayling IM, Beck NE, Ilyas M, *et al*: The *APC* variants I1307K and E1317Q are associated with colorectal tumors, but not always with a family history. *Proc Natl Acad Sci USA* 95: 10722-10727, 1998.
19. Lamlum H, Al Tassan N, Jaeger E, *et al*: Germline *APC* variants in patients with multiple colorectal adenomas, with evidence for the particular importance of E1317Q. *Hum Mol Genet* 9: 2215-2221, 2000.
20. Azzopardi D, Dallosso AR, Eliason K, *et al*: Multiple rare nonsynonymous variants in the adenomatous polyposis coli gene predispose to colorectal adenomas. *Cancer Res* 68: 358-363, 2008.
21. Cui DH, Jiang KD, Jiang SD, Xu YF and Yao H: The tumour suppressor adenomatous polyposis coli gene is associated with susceptibility to schizophrenia. *Mol Psychiatry* 10: 669-677, 2005.
22. Egan JB, Jacobs ET, Martínez ME, Gerner EW, Jurutka PW and Thompson PA: Presence of a TA haplotype in the *APC* gene containing the common 1822 polymorphism and colorectal adenoma. *Cancer Res* 68: 6006-6013, 2008.
23. Miller SA, Dykes DD and Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 16: 1215, 1998.
24. Umar A, Boland CR, Terdiman JP, *et al*: Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch Syndrome) and microsatellite instability. *J Natl Cancer Inst* 96: 261-268, 2004.
25. Ribas G, Milne RL, Gonzalez-Neira A and Benitez J: Haplotype patterns in cancer-related genes with long-range linkage disequilibrium: no evidence of association with breast cancer or positive selection. *Eur J Hum Genet* 16: 252-260, 2008.