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Association of Common Polymorphisms in Inflammatory Genes Interleukin (*IL*)*6*, *IL8*, Tumor Necrosis Factor α, *NFKB1*, and Peroxisome Proliferator-activated Receptor γ with Colorectal Cancer[1,2]

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ABSTRACT

Animal models and epidemiological observations suggest that a continuous inflammatory condition predisposes to colorectal cancer (CRC), but the roles of different elements participating in inflammatory responses have been little investigated in relation to CRC. We have studied the association between single nucleotide polymorphisms in the interleukin (*IL*)-*6* (−174 G>C), *IL8* (−251T>A), tumor necrosis factor α (−308G>A), and *PPARG* (Pro12Ala) genes and the risk of CRC in a group of 377 cases and 326 controls from Barcelona, Spain. These genes are known to be important for inflammation of the colorectum and common allelic variants have been shown to have a biological effect. The *PPARG* Ala12 and *IL8*-251A genotypes are associated with reduced risk of disease (0.56, 95% CI, 0.37–0.85, $P = 0.0056$, and 0.70, 95% CI, 0.50–0.99, $P = 0.043$, respectively), whereas the *IL6*–174C genotype is associated with increased risk (1.53, 95% CI, 1.12–2.09, $P = 0.0073$). We also studied a single nucleotide polymorphism in intron 11 of the *NFKB1* gene (rs1020759), which probably lacks any functional role, and found no significant association with the disease. This is the first report that *IL6*, *IL8*, and *PPARG* genes are important in relation to inflammation-related risk of sporadic CRC.

INTRODUCTION

Inflammation is a complex response, at the cell and tissue level, to a variety of stimuli, including heating, trauma, viral/bacterial infections, and endotoxemia, and is often a consequence of immune system activity and wound healing (1–3). A persistent state of inflammation is thought to produce chronic damage leading to atherosclerosis (4), neurodegenerative disorders (5), and certain types of cancer (6). Examples include the relationships between bronchitis/emphysema and lung cancer (7, 8), schistosomiasis and bladder cancer (9), asbestos fiber inhalation and mesothelioma (10), chronic esophagitis and carcinoma of the esophagogastric junction (11), *Helicobacter pylori* infection and gastric cancer (12), and IBD[5] and CRC (13, 14). Moreover, precursor lesions of CRC, whether adenomas or polyps, often have inflammatory histological features (14, 15). Inflammation favors tumorigenesis by stimulating angiogenesis (16), damaging DNA (17, 18), and chronically stimulating cell proliferation (19, 20). Proinflammatory genes have been shown also to be important for the maintenance and progression of CRC (21). These findings may suggest reasons why regular use of NSAIDs such as aspirin or ibuprofen is associated with a 40–50% decrease in relative risk for colon cancer (22).

In normal colon and rectum, the bacterial flora keeps the mucosa in a continuous state of low-grade inflammation and stimulates release of proinflammatory cytokines by the immune cells (14, 23). Cytokines bind to specific receptors and activate the NF-κB transcription factor signal pathway in the epithelial cells as well as in the cooperating immune cells, leading to up-regulation of the *PTGS2*, *IL4*, *IL6*, and *IL-8* genes (14). Cyclooxygenase and arachidonate metabolites have been shown to inhibit apoptosis (24) and enhance angiogenesis (25), whereas IL-8 and IL-6, via the STAT3 intracellular signal pathway, have proinflammatory activity in the intestine (26, 27). PPARγ, a nuclear receptor transcription factor, may also play a role in bowel inflammation (28). Recently, natural ligands and drug agonists of PPARγ were shown to reduce intestinal inflammation in IBD patients and in rodents via inhibition of both the NF-κB and STAT3 pathways (29, 30). PPARγ has been found to contain inactivating mutations in sporadic colorectal carcinomas (31), and colorectal carcinoma cell lines treated with PPARγ ligands showed decreased DNA synthesis, G_1 cell cycle arrest, and increased expression of markers of differentiation (32). In addition, chemically induced colonic inflammation and formation of aberrant crypt foci (early precursor lesions of colon cancer) were diminished in animal models by administration of PPARγ ligands (33).

SNPs in the *IL6*, *IL8*, *PPARG*, and *TNF* (coding for TNF-α) genes have been shown to be related to changes in biological functions of inflammation pathways. *IL6* −174 G>C is a SNP affecting the transcription of the gene, *i.e.*, altering the final levels of IL-6 released (34, 35). The C allele is associated with several inflammatory-related conditions, including increased plasma levels of C-reactive protein (36), a steeper release of IL-6 after coronary artery bypass surgery (37), asymptomatic carotid artery atherosclerosis (38), less favorable prognosis after abdominal aortic aneurysms (perhaps because of increased inflammation after aortic trauma; Ref. 39), and poorer survival after sepsis (38). Other changes such as increased systolic blood pressure (40) lower bone resorption (38), as well as reduced risks of AIDS-associated Kaposi's sarcoma (41), have also been described.

The A-allele of a SNP in the promoter region of the *TNF* gene (−308 G>A) is associated with higher expression *in vitro* and *in vivo* of TNF-α (42) and with susceptibility to cerebral malaria, celiac disease, and other immune-related disease (38). In one study, the A-allele of the SNP −251 T>A in the *IL8* gene was found to be related to higher *in vitro* levels of IL-8 after stimulation with lipo-

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[5] The abbreviations used are: IBD, inflammatory bowel disease; SNP, single nucleotide polymorphism; IL, interleukin; TNF, tumor necrosis factor; PPARG, peroxisome proliferator-activated receptor γ; CI, confidence interval; OR, odds ratio; NSAID, nonsteroidal anti-inflammatory drug; STAT3, signal transducer and activator of transcription 3; NF-κB, nuclear factor κB; CRP, C-reactive protein; CRC, colorectal cancer; BMI, body mass index.

polysaccharide and associated with respiratory syncytial virus bronchiolitis in children (43).

PPARG has a polymorphism in the coding region (34C>G) that results in the amino acid change Pro12Ala (44). The G34 (Ala12) allele is associated with reduced risk of type 2 diabetes (45), improved insulin sensitivity (46, 47), and lower risk of renal cell carcinoma (48).

In this study, we investigated the roles of SNPs *IL6* −174 G>C, *TNF* −308 G>A, *IL8* −251 T>A, and *PPARG* 34 C>G as host risk factors for CRC in a case-control study. We also investigated the role of a frequent, but functionally untested, SNP in intron 11 of *NFKB1* (dbSNP ID rs1020759) because this gene is central for inflammation, and no functional polymorphism on it has yet been described. The effects of the polymorphisms were studied as main effects and as interactions with various other variables, including dietary factors and intake of NSAIDs.

SUBJECTS AND METHODS

Study Population. A hospital-based case-control study was conducted to assess gene-environment interactions in relation to CRC risk. Cases were patients with a new diagnosis of CRC attending an university hospital in Barcelona, Spain, between January 1996 and December 1998. All cases had histological confirmation of their tumor diagnosis. During the study period, a total of 523 cases was diagnosed with CRC in the hospital. This study includes those 377 (72%) who could be interviewed and who provided biological samples of sufficient quality for genetic analysis. Refusals were 2%, whereas 14% could not be interviewed because they either had died, had mental or some other impairment, or were released without being approached and could not be traced. Finally, 12% were interviewed but did not provide biological samples. These lost cases were similar to those included with respect to age, sex, tumor location, and extent.

Controls were randomly selected among patients admitted to the same hospital during the same period. To avoid selection bias, the criterion for inclusion of controls was that the reason for the current admittance to the hospital should be a new disease (not previously diagnosed) for that patient. This criterion was used to avoid inclusion of patients with chronic diseases who might be repeatedly admitted to hospital and modify their habits because of their disease. This procedure paralleled the criterion for cases who were also newly diagnosed incident cases. Sex and broad age groups were used as stratifying criteria for frequency matching. Both cases and controls had to have good mental condition and be able to see and hear and follow an interview. From the daily patient admission lists, candidate controls were approached, and if they met these criteria, they were invited to participate. Among 470 selected controls, a total of 326 (69.4%) was analyzed in this study. Refusals were 7%, whereas 5% could not be interviewed because of mental or other impairment. Finally, 87 (18.6%) were interviewed but did not provide a blood sample. From a genetic point of view, we consider the hospitalized controls as being representative of the general Spanish population because they came from very different hospital departments and included very different diseases. Moreover, it was recently reported in a study of 15,000 hospitalized controls that the frequencies of genetic polymorphisms did not differ from those of population controls (49). No restriction criterion was imposed regarding the diagnosis of controls except those previously mentioned. The distribution of controls by diagnostic group was as follows: internal medicine 22%; acute surgery 19%; urology 17%; traumatology 15%; gastroenterology 16%; and circulatory or respiratory 11%. Sixty controls (18%) had a diagnosis of inflammatory conditions that might be related to the studied polymorphisms: IBD ($n = 2$); peptic ulcer ($n = 2$); pancreatitis ($n = 24$); cholecystitis ($n = 16$); arthritis ($n = 12$); and diverticulitis ($n = 4$). We compared the distribution of genotypes in this group and found no difference from the rest of the controls. Also, an analysis of the data excluding these controls yielded essentially the same results. Thus, we decided not to exclude any control that had been selected.

All subjects were informed and gave written consent to participate in the study and to allow their biological samples to be genetically analyzed, according to the Helsinki declaration. The study protocol was cleared by the ethical committee of the hospital.

Interviews. Cases and controls were personally interviewed by trained personnel using a structured questionnaire to determine demographic characteristics and potential risk factors for CRC. For each subject, age and sex were recorded. A dietary history questionnaire, previously developed and validated in the framework of the European Prospective Investigation on Nutrition and Cancer study (50), focused on average food consumption 1 year before the diagnosis of disease. The questionnaire recorded individual foods. For the analysis, posthoc-selected food groups (vegetables, fruits, and so on) were created by adding the consumption in grams of specific foods. Nutrient intakes were estimated with food composition tables developed *ad hoc* for the European Prospective Investigation on Nutrition and Cancer study (51). To adjust for total energy intake, nutrient densities were calculated by dividing nutrient intake by total calories estimated from macronutrients plus alcohol. Supplemental vitamin intake is rare in this community and was not recorded. Self-reported weight and height were recorded for all individuals. Real weight and height were measured during the interview for 40% of the sample. Because the agreement between measured and reported values was very good (Pearson's $r = 0.97$), reported values were used for individuals if measurements were unavailable. Reported weight 10 years before interview was also recorded. BMI at that time was calculated assuming no change in height.

Lifelong long-term (at least 6 consecutive months) drug use was included in the questionnaire. An initial open question was followed by a list of 20 chronic diseases that usually are treated pharmacologically and their treatments were recorded. No drug list was used. For each exposure, the ages at initial use and cessation were recorded, and cumulative duration was computed. Drugs were grouped using the anatomical therapeutic chemical classification, and the focus in this analysis was on NSAIDs. Other relevant risk factors explored were smoking, alcohol, and family history of cancer. For tobacco and alcohol, life-long history was recorded. We defined as an alcohol drinker a person consuming a minimum of one standard unit (equivalent to 10 g of pure alcohol)/week. Average daily consumption and duration were calculated by summing the different exposure patterns during life.

Biological Samples. A blood sample was obtained from each case and control. DNA was extracted by the standard phenol/chloroform method. In cases, DNA was also extracted from fresh frozen tumor tissue. Tumors were investigated for genetic alterations on k-ras and p53. The detection and characterization of mutations at codons 12 and 13 of the K-*ras* was performed by PCR/single-strand conformation polymorphism.

Overexpression of the p53 protein was determined immunohistochemically, using commercially available antibodies [monoclonal mouse IgG antibody for p53 (ab-6) Pantropic; Oncogene]. Cases were classified as overexpressing p53 when >20% of the cells were stained. Two pathologists read the slides, and a consensus was reached for initially discordant cases.

SNP Selection. The aim of this work was to investigate several candidate genes conferring susceptibility to CRC. Firstly, we identified genes of interest having a role in inflammation and possibly also in other mechanisms important for tumorigenesis such as cell differentiation (*PPARG*) or angiogenesis (*IL6*). We then chose the most representative SNP for each gene, according to two criteria: function and frequency. We selected those SNPs for which there was information on the function (such as A-308 of *TNF*) or which had already been shown to be positively associated with more than one outcome in case-control association studies (such as higher systolic blood pressure for C-174 of *IL6*). In terms of frequency, we excluded polymorphisms with a frequency < 10% because the statistical power of a study with our sample size would be inadequate with polymorphisms of lower frequency. Moreover, we did not include polymorphisms too far from the coding region that would be unlikely to have a functional role for the gene.

Thus, our final selection included the *IL6* (−174 G>C), *IL8* (−251T>A), *TNF* (−308G>A), and *PPARG* (Pro12Ala) polymorphisms. In addition, we included one SNP for the *NFKB1* gene because this transcription factor is crucial in the inflammatory response. Because no SNPs with known function have been reported for this gene, we chose one of the most frequent in intron 11.

SNP Genotyping. Samples from cases and controls were randomized and mixed on PCR plates so that an equal number of cases and controls could be analyzed simultaneously. Genotyping was carried out with the 5′ nuclease assay by minor groove binder probes fluorescently labeled with FAM or VIC and using the protocol recommended by the supplier (Applied Biosystems, Foster City, CA). Reactions were run in 96-well plates on a Tetrad DNA

Appendix 1 *Probes and primers used for SNP genotyping*

IL-6 174 G>C (rs1800795)
VIC-TCTTGCGATGCTAAA
FAM-TCTTGCCATGCTAAA
Forward primer: TGACGACCTAAGCTGCACTTTTC
Reverse primer: GGGCTGATTGGAAACCTTATTAAGA
TNF − 308 G>A (rs3091256)
VIC-CCGTCCCCATGCC
FAM-CCGTCCTCATGCC
Forward primer: GAAATGGAGGCAATAGGTTTTGAG
Reverse primer: GGCCACTGACTGATTTGTGTGTAG
IL-8 − 251 T>A (rs4073)
VIC-CATACAATTGATAATTCA
FAM-CATACATTTGATAATTCA
Forward primer: TAAAATACTGAAGCTCCACAATTTGG
Reverse primer: ATCTTGTTCTAACACCTGCCACTCT
PPARG 34 C>G (rs1801282)
VIC-TTCTGGGTCAATAGG
FAM-CTTTCTGCGTCAATAG
Forward primer: TTATGGGTGAAACTCTGGGAGATT
Reverse primer: TGCAGACAGTGTATCAGTGAAGGA
NFKB1 intron 11 (rs1020759)
VIC-CCCATCGGTTATATC
FAM-CCCCATCAGTTATATC
Forward primer:
GTCTGCAGTGAACAGTAATGAGTATAGCT
Reverse primer:
GGAGAAACTTTGACTTACTTATCAAATATGTT

Engine PCR machine (MJ Research, Waltham, MA) and read in a TaqMan 7900HT sequence detection system (Applied Biosystems). The probes and primers for the genotyping reactions are reported in appendix 1.

Data Analysis. Each polymorphism was tested in controls to ensure that it fits Hardy-Weinberg equilibrium. To test the hypothesis of association between genetic polymorphisms and CRC, multivariate methods based on logistic regression analyses were used. When cases were subdivided into groups, polytomous logistic regression was used, comparing each group of cases with the whole set of controls. Cases were subdivided based on the location of the tumor, the k-ras mutation status or overexpression of p53. ORs and 95% CIs were calculated for each group compared with the class with the lowest level of exposure (set as having risk = 1). For polymorphisms, homozygosity for the more frequent allele among controls was set as the reference class. Tests for linear trend of ORs were calculated using the categorized variable as quantitative after assigning a linear score to each ordered category. For polymorphisms, the homozygote for the more frequent allele (reference) was given score 1, the heterozygote score 2, and the homozygote for the rarer allele score 3. *P*s were derived from likelihood ratio tests. Tests for interactions were performed comparing the change in deviance (−2·log likelihood) between the model with the main effects and the model that also included the interaction term (product of the main effects). Analyses were performed under both a codominant model (three genotypes separated) and a dominant model (heterozygous grouped with the homozygous for the rarer allele) to increase the statistical power. All analyses were adjusted for age and sex. When interactions with dietary variables were studied, the analyses were adjusted also for BMI and total caloric intake.

A significance level of 5% (two-sided) was used for the analyses. Because many tests were done, this significance level may result in false positive results, but this was considered acceptable for an exploratory study (52).

RESULTS

The relevant characteristics of the subjects are shown by case/control status in Table 1. In general, the CRC patients used less NSAIDs, had higher daily caloric intake, consumed less fruit and vegetables, and drank more alcohol than the controls. In our population, consumption of NSAIDs was associated with a relative risk of 0.70 (95% CI, 0.52–0.94). Ethanol is an irritant for the digestive tract (53) and is moderately associated with cancer of the colon and rectum (54). We found ever drinking alcohol to be associated with a relative risk of 1.31 (95% CI, 0.93–1.84) as a main effect. Regular drinkers of alcohol (at least one standard unit, equivalent to 10 g of pure alcohol/week) during >40 years relative to never-drinkers had an OR of 1.54 (95% CI, 1.03–2.31, $P_{trend} = 0.036$).

PPARG *Polymorphism.* The ORs for the associations for the polymorphisms considered are shown in Table 2, both as separated (codominant model) and grouped (dominant model) genotypes. The G-allele (Ala12) of *PPARG* was significantly associated with a reduced risk of CRC. When genotypes were grouped, the risk was 0.56 (95% CI, 0.37–0.85, $P = 0.0056$). This association was essentially the same when colon and rectum were considered separately. Moreover, *PPARG* genotypes appeared to interact with the assessed dietary intake of vitamin A. The main effect of vitamin A is protective: the OR for the highest tertile of consumption (>610 μg/day) compared with the lowest tertile (<400 μg/day) was 0.67 (95% CI, 0.47–0.95, $P_{trend} = 0.023$). The relative protective effect of *PPARG* Ala12 was more evident in those consuming lower amounts of vitamin A (OR = 0.25; 95% CI, 0.12–0.53) than in those with high intake (OR = 1.08; 95% CI, 0.53–2.2). The *P* for the interaction was 0.014 and for the interaction considering the linear trend in vitamin A consumption was 0.005.

In this study, *PPARG* Ala12 was associated with BMI in cases but not in controls. Among cases, the OR of having a BMI ≥ 25 kg/m^2

Table 1 *Characteristics of colorectal cancer patients and control subjects*

	Cases (n = 377) %	Controls (n = 326) %
Males	59.7	53.1
Females	40.3	46.9
Age		
<58	21.7	26.4
58–67	24.7	26.7
68–75	29.2	20.2
>75	24.4	26.7
Rectal cancer	36.8	
Colon cancer	63.2	
Dukes extent		
A1-B1-B2	46.5	
C1-C2	34.4	
D	19.1	
Mutations		
TP53	77.0	
ras	36.7	
Smoking		
Nonsmokers	53.2	55.9
Ex-smokers	31.6	26.1
Smokers	15.2	18
Alcohol drinkers (>10 g/week)		
Never	34.6	43.3
<40 years	31.7	30.5
>40 years	35.3	25.0
Use of drugs (weekly during >6 months)		
Acetylsalicylic acid	16.1	17.0
Other NSAIDs	5.8	14.5
Paracetamol	10.5	17.4
Antiulcer	16.9	13.6
Laxatives	17.2	14.5
Family history of cancer in first degree relatives		
All cancers	42.0	34.3
Colorectal cancer	11.7	3.8
Caloric intake		
<1688	29.2	37.9
1688–2246	34.4	31.9
≥2246	36.3	30.2
BMI (at diagnosis)		
>25	44.5	38.5
25–29	41.5	39.4
≥30	14.0	22.1
BMI (10 years before diagnosis)		
>25	36.2	37.5
25–29	43.8	43.0
≥30	20.0	19.5
Vitamin A intake (μg/day)		
<400	37.3	28.9
400–610	34.2	32.7
>610	28.5	38.4

Table 2 *ORs for colorectal cancer by genotypes under investigation (main effects)*

	Controls	All cases		Rectal cancer		Colon cancer	
	n^a	n^a	OR^c (95% CI)	n^a	OR^c (95% CI)	n^a	OR^c (95% CI)
PPARG							
C/C	243	311	1.00	111	1.00	200	1.00
C/G	61	46	0.57 (0.37–0.87)	15	0.53 (0.29–0.98)	31	0.59 (0.37–0.96)
G/G	5	3	0.45 (0.10–1.91)	3	1.22 (0.28–5.24)	0	
Trend test P^b			0.0056		0.13		0.0068
PPARG (grouped)							
C/C	243	311	1.00	111	1.00	200	1.00
C/G + G/G	66	49	0.56 (0.37–0.85)	18	0.59 (0.33–1.04)	31	0.55 (0.34–0.88)
P			0.0055		0.066		0.012
IL-6							
G/G	145	133	1.00	44	1.00	89	1.00
G/C	133	180	1.50 (1.08–2.09)	72	1.81 (1.16–2.82)	108	1.35 (0.93–1.96)
C/C	33	48	1.65 (0.99–2.74)	17	1.69 (0.85–3.33)	31	1.63 (0.93–2.87)
Trend test P^b			0.011		0.023		0.044
IL-6 (grouped)							
G/G	145	133	1.00	44	1.00	89	1.00
G/C + C/C	166	228	1.53 (1.12–2.09)	89	1.78 (1.16–2.73)	139	1.4 (0.99–2)
P			0.0073		0.0078		0.059
TNF							
G/G	234	278	1.00	105	1.00	173	1.00
G/A	76	80	0.87 (0.60–1.25)	28	0.82 (0.50–1.35)	52	0.90 (0.59–1.35)
A/A	10	5	0.40 (0.13–1.19)	1	0.21 (0.03–1.69)	4	0.51 (0.15–1.68)
Trend test P^b			0.13		0.13		0.30
TNF (grouped)							
G/G	234	278	1.00	105	1.00	173	1.00
G/A + A/A	86	85	0.81 (0.57–1.16)	29	0.75 (0.46–1.22)	56	0.85 (0.57–1.26)
P			0.25		0.24		0.42
IL-8							
T/T	83	117	1.00	39	1.00	78	1.00
T/A	170	167	0.66 (0.46–0.95)	61	0.74 (0.46–1.20)	106	0.62 (0.42–0.93)
A/A	55	68	0.83 (0.53–1.32)	28	1.05 (0.58–1.91)	40	0.72 (0.43–1.22)
Trend test P^b			0.26		1		0.12
IL-8 (grouped)							
T/T	83	117	1.00	39	1.00	78	1
T/A + A/A	225	235	0.70 (0.5–0.99)	89	0.81 (0.52–1.28)	146	0.65 (0.44–0.95)
P			0.043		0.38		0.025
NFKB1							
G/G	118	146	1.00	50	1.00	96	1.00
G/A	154	150	0.78 (0.56–1.08)	56	0.85 (0.54–1.33)	94	0.74 (0.51–1.08)
A/A	34	49	1.14 (0.69–1.89)	20	1.38 (0.72–2.64)	29	1.01 (0.57–1.79)
Trend test P^b			0.82		0.64		0.52
NFKB1 (grouped)							
G/G	118	146	1.00	50	1.00	96	1.00
G/A + A/A	188	199	0.84 (0.61–1.16)	76	0.94 (0.62–1.44)	123	0.79 (0.55–1.13)
P			0.29		0.79		0.19

[a] No. of subjects.
[b] For the trend test, the homozygote for the more frequent allele (reference) was given score 1, heterozygote score 2, and the homozygote for the rare allele score 3. All statistical tests were two-sided.
[c] OR, with 95% CI, adjusted for sex and age.

was 2.03 (95% CI, 0.99–4.12) and the OR of BMI ≥ 30 was 2.83 (95% CI, 1.17–6.87, $P_{trend} = 0.014$) for individuals with the Ala12 variant. Among controls, the OR for BMI ≥ 30 was 1.00 (95% CI, 0.47–2.13, $P_{trend} = 0.99$). Because cases tend to lose weight because of the disease, this association could be interpreted as suggesting that *PPARG* Ala12 was related to less weight loss. This interpretation is supported by the observation that the reported weight 10 years before the disease showed no association. At that time, *PPARG* Ala12 had an OR for obesity (BMI > 30) of 0.78 (95% CI, 0.32–1.92).

***IL6* Polymorphism.** The allele *IL6* −174C was associated with increased risk of CRC. The OR for the C/C genotype was 1.65 (95% CI, 0.99–2.74, $P_{trend} = 0.011$). This association was seen both under a codominant model as well as when genotypes were grouped for both cancer of the colon and cancer of the rectum. To our knowledge, this is the first time that this polymorphism of the regulatory region of *IL6* has been associated with cancer.

When investigated for interactions, the effect of the *IL6* polymorphism −174C contributed multiplicatively to the relative risk independently of most of the variables analyzed, including the interaction between *IL-6* and *PPARG* genotypes. However, interestingly, the effect of alcohol drinking (associated with risk of cancer as a main effect) was evident only in the subgroup of C-carriers (OR = 2.19, 95% CI, 1.3–3.7, $P = 0.038$; Table 3). Moreover, when we stratified for use of NSAIDs, we found the association between the C-allele and cancer only in the subgroup of those who habitually did not take

Table 3 *ORs for interactions between* IL-6 *genotypes (grouped) and colorectal cancer stratified by alcohol consumption and NSAIDs drug intake*

	Non drinkers		Drinkers	
	n^a	OR^b (95% CI)	n^a	OR^b (95% CI)
IL-6				
G/G	51/58	1	82/87	1.08 (0.63–1.86)
G/C + C/C	74/83	1.03 (0.63–1.69)	154/83	2.19 (1.3–3.7)
Interaction *P*=	0.038			
	No habitual use of NSAIDs		Habitual use of NSAIDs	
	n^a	OR^b (95% CI)	n^a	OR^b (95% CI)
IL-6				
G/G	91/104	1	42/41	1.15 (0.68–1.93)
G/C + C/C	166/96	2.02 (1.38–2.95)	62/70	1.02 (0.65–1.61)
Interaction *P*=	0.018			

[a] No. of subjects in cases/controls.
[b] OR, with 95% CI, adjusted for sex, age, calories/day, and BMI.

NSAIDs. For carriers of the C-allele, the use of NSAIDs halved the risk from 2.02 (95% CI, 1.38–2.95) to 1.02 (95% CI, 0.65–1.61; Table 3).

***IL8, TNF*, and *NFKB1* Polymorphisms.** The allele A of *IL8* in position −251 was associated with decreased risk of cancer, but this was significant only when genotypes were grouped. The A-allele of *TNF* in position −308 was also associated with a decreased risk of cancer, but because of the low frequency of this variant, the difference was not significant. No effect of *NFKB1* polymorphism was found (Table 2).

Interactions with Tumor Characteristics. An exploratory analysis of the risk of CRC in relation to the polymorphisms was performed by stratifying the cases by subsite (rectum, right colon, and left colon), tumor stage (Dukes A/B, C, and D), and genetic alterations (K-*ras* mutation status and p53 overexpression). All of the main effect associations previously reported were essentially the same for the different case groups analyzed.

DISCUSSION

In this study, we explored the role of polymorphisms in genes related to inflammation in CRC. We found that a polymorphism in the promoter of the *IL6* gene is associated with a significantly increased risk of CRC, whereas polymorphisms in the *PPARG* and *IL8* genes were related to reduced risk. In general, heterozygotes showed intermediate risks, and the associations were consistent in colon and rectum.

The PPARγ Ala12 variant associates with a reduced CRC. A number of plausible mechanisms may account for this protective effect. *PPARG* Ala12 has been associated with higher cellular sensitivity to insulin (46, 47). This could lead to a reduced level of circulating insulin through a mechanism involving reduced release of free plasmatic fatty acids by adipocytes (28). Notably, a decreased plasma level of insulin has been associated with reduced risk of CRC (55). The fact that PPARγ is expressed in the large intestine mostly as isoform PPARγ1 (the first exon is spliced out; Ref. 45) reinforces the idea that the protective effect of the polymorphism could be mediated by the adipocytes, where the isoform γ2 (retaining the first exon) is the most abundant (28). PPARγ could also act by other mechanisms. Activation of the receptor by artificial ligands has been shown to inhibit the NF-κB and STAT3 inflammation pathways (attenuating IBD and related conditions; Refs. 29, 30), to induce the differentiation of CRC cells (32), to trigger apoptosis in breast (56), colon (57), prostate (58), and lung (59) cancers, to decrease DNA synthesis, and to cause G_1 cycle arrest of colorectal and pancreatic cancer cell lines (60). The fact that Ala12 variant is associated with reduced activity in the absence of ligands (28) does not provide support for the latter hypothesis. However, little is known about the behavior of PPARγ in the presence of its natural ligands, and it would not be surprising if Ala12 were more active in that situation.

Interestingly, we also found that the Ala12 variant was more protective in subjects who had low dietary intake of vitamin A. Although this interaction may have arisen by chance, because of the many interactions explored, it could be a result of formation of an active heterodimeric DNA binding complex of PPARγ with the retinoid X receptor (61). These hypotheses deserve additional research, and functional studies need to be performed. In any case, *PPARG* appears to be a suitable candidate gene for CRC susceptibility through its pleiotropic activity.

In our study, the C-174 allele of *IL6* was associated with increased risk of CRC and also increased systolic pressure (data not shown but confirming a previous report; Ref. 40). IL-6 could be related to CRC by affecting the low-grade inflammation status of the intestine. IL-6 is the most important cytokine in modulating cross-talk between the intestinal epithelial cells and the neutrophils activated by the bacteria populating the intestinal lumen (27). IL-6 is also important at the systemic level both as a pro- and anti-inflammatory cytokine: it targets the liver to increase the release of C-reactive protein and stimulates fibroblasts and epithelial cells to secrete IL-1β receptor antagonists (IL1-Ra; Refs. 62, 63).

Functional studies have not fully elucidated the biological role of the substitution −174 G>C. The C-174 allele was associated *in vitro* and *in vivo* with alternatively lower or higher levels of expression of IL-6 (34–36). From all these studies, it is known that the promoter harbors binding sites for multiple transcription factors and the regulation of *IL6* is very complex (64). The polymorphism could cause different effects according to the cell type and the stimulus applied. The C-174 allele could cause increased inflammation for colorectal cells in response to activated neutrophils (27). It could also be associated with reduced release of IL-6 at the systemic level, thereby down-regulating the IL1-Ra-mediated anti-inflammatory activity. Interestingly, the C-174 genotype was associated with an increased risk of CRC only in those subjects who did not habitually take NSAIDs. This would support the hypothesis that C-174 genotypes are associated with an increased level of basal inflammation reducible by the use of NSAIDs. IL-6 could also affect a tumor's ability to form new blood vessels, but its role in the angiogenesis is not clear (65, 66).

When we analyzed *IL8*, we observed a reduced risk of cancer for the A-251 allele. There is limited information at present on the function of the *IL8* polymorphism. This SNP has been found to have an effect in changing the *in vitro* levels of IL-8 and has been associated with respiratory syncytial virus bronchiolitis in children (43). However, inflammation is a highly regulated process, and the role of this polymorphism in the intestine is not easily transposable.

Among the other polymorphisms, we found a trend of reduced risk in *TNF* A-308 allele carriers. CRC was not previously found to be associated with −308 SNPs alone (67) or in combination with another rarer SNP at −238 (68). One study showed an association between a microsatellite (locus TNFd) and CRC (69). However, the locus is 8–10 kbp downstream of the gene and is unlikely to affect *TNF* functionally. The lack of a statistically significant association precludes any possible hypothetical involvement of A-308 in colorectal tumorigenesis.

Finally, we did not find any association between *NFKB1* polymorphism and CRC. No functional polymorphisms of *NFKB1* have been described. The dbSNP[6] reports 95 polymorphisms mapping within >11 kbp of the genomic region. Among these, only two are in the coding sequence, but they result in synonymous changes and have not been validated experimentally. It is likely that this transcription factor is highly conserved and that mutations are eliminated by the evolutionary pressure. It is also likely that the intronic SNP we studied does not change the function of *NFKB1*, and the lack of association is not surprising.

We are aware that in any case-control study, there are potential limitations and that the use of hospital controls is not ideal. We tried to minimize potential bias (*e.g.*, recall bias) by careful design and by use of validated questionnaires administered by trained interviewers. The use of hospital controls probably has minimal effect on the allele frequencies, as previously shown (49). Moreover, if proinflammatory alleles were related to diseases other than CRC, this would increase their prevalence among the controls and bias the ORs toward the null. Another possible bias could be represented by the cases lacking interviews or biological material. However, these cases were similar

[6] Internet address: http://www.ncbi.nlm.nih.gov/SNP/.

in demographic and tumor characteristics to those included, and no associations of the polymorphisms with these characteristics have been found relevant.

Another possible limitation is the lack of haplotype reconstruction. Haplotypes are considered more powerful to detect susceptibility alleles than individual polymorphisms. However, in this study, we chose polymorphisms causing an actual alteration of function. In this case, it has been shown that the functional polymorphism gives the highest association values as compared with the haplotypes (70).

In conclusion, we report here, for the first time, the association of common SNPs in the *PPARG*, *IL6*, and *IL8* genes with CRC. Studies should be undertaken to additionally investigate the molecular mechanisms leading to the disease; if the relationships observed in this study are confirmed, they might form a basis for specific chemoprevention programs for people at increased risk for CRC.

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