Influence of the C161T but not Pro12Ala polymorphism in the peroxisome proliferator-activated receptor-gamma on colorectal cancer in an Indian population

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The aim of the present study was to investigate associations between Pro12Ala and C161T polymorphisms in the peroxisome proliferator-activated receptor-gamma (*PPAR-γ*) gene and colorectal cancer (CRC) risk. We recruited 301 newly diagnosed CRC patients and 291 healthy control subjects at the Madras Cancer Institute in Chennai, India, from 1999 to 2001. Genotypes of the Pro12Ala and C161T polymorphisms were determined using the PCR-RFLP method. After adjustment for age, sex, smoking habit, family history and family income, an increased risk of CRC was observed for the C/T + T/T genotype compared to the C/C genotype of the C161T polymorphism (odds ratio = 1.61, 95% confidence interval: 1.10–2.36), whereas no significant association was found for Pro12Ala (odds ratio = 1.06, 95% confidence interval: 0.70–1.61). Analysis with estimated haplotypes showed a significant difference in haplotype frequencies between cases and controls (χ^2 = 11.62, *P* = 0.009, d.f. = 3). The relationship between the two polymorphisms and CRC risk was not significantly modified by dietary intake of fish. Although the biological mechanisms of the observed association remain to be elucidated, our findings suggest that the C161T polymorphism of the *PPAR-γ* gene is related to risk of CRC. Further research is needed to investigate functional implications of polymorphisms of the *PPAR-γ* gene in CRC development. (*Cancer Sci* 2005; 96: 507–512)

The peroxisome proliferator-activated receptor-gamma (*PPAR-γ*), a member of the nuclear hormone receptor super family, plays an important role in differentiation of adipocytes, lipid metabolism, insulin sensitivity, atherogenesis and immune regulation.[1–4] Recently, *PPAR-γ* has been implicated in the pathogenesis of colorectal cancer (CRC) in animal models and clinical studies. Colon cancer cells have been shown to express *PPAR-γ* at high levels, and somatic loss-of-function mutations have been identified.[5,6] *In vitro* studies have shown that ligand activation of *PPAR-γ* could inhibit the nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3) inflammation pathways and cell growth, induce apoptosis and promote differentiation in colon, breast and prostate cell lines.[7–10] Furthermore, a *PPAR-γ* ligand was found to inhibit tumor growth in a xenograft model of colon cancer, and decrease premalignant intestinal lesions in mice treated with the chemical carcinogen azoxymethane.[11,12] In addition, increased susceptibility of *PPAR-γ* heterozygote knockout mice to colorectal carcinogenesis has been reported.[13] These observations raise the exciting hypothesis that *PPAR-γ* is a tumor suppressor gene in colorectal carcinogenesis. However, one study failed to find any *PPAR-γ* mutations in colon cancer samples, and another showed that administration of *PPAR-γ* ligands to Min mice resulted in development of more advanced colon cancers.[14,15]

The human *PPAR-γ* gene exists in three isoforms due to alternative promoters and differential splicing. PPAR-γ1 and PPAR-γ3 proteins are almost identical and are encoded by exons 1–6, whereas PPAR-γ2 has 28 additional amino acids at its N-terminus, encoded by the *PPAR-γ2*-specific exon B. The PPAR-γ1 and PPAR-γ3 isoforms are expressed in large intestinal, kidney and adipose tissues, while PPAR-γ2 exists exclusively in adipose tissue.[16,17] Common structural polymorphisms that have been detected in the *PPAR-γ* gene include a proline to alanine substitution (34C > G), located at codon 12 (Pro12Ala) of *PPAR-γ2*-specific exon B,[18] which reduces the promoter affinity by approximately 50%, and both ligand-independent and ligand-dependent *PPAR-γ* transactivation.[2] Another common polymorphism in exon 6 at nucleotide 161 results in a silent substitution from C to T (C161T).[3]

Recently, Landi *et al*. showed the Pro12Ala polymorphism to be related to a reduced CRC risk in a Spanish population.[19] Gong *et al*. also reported a decreased risk of colorectal adenomas associated with the 12Ala allele in *PPAR-γ*, with marginal significance.[20] Siezen *et al*. demonstrated a protective effect of the C/T genotype of the C161T polymorphism with reference to colorectal adenomas.[21] On the one hand, recent studies found

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no association between the Pro12Ala polymorphism and the risk of colorectal adenomas, prostate cancers or breast cancers, and there is some evidence that the C161T polymorphism may in fact be related to an increased risk of endometrial and prostate cancers, as well as glioblastoma multiforme.[21–25] Thus, the two common polymorphisms in the *PPAR*-γ gene may play a role in the etiology of cancer, but the results have been equivocal.

We therefore conducted the present study in an Indian population. Moreover, previous studies have shown that n-3 polyunsaturated fatty acids from fish may induce apoptosis in colon cells, and *PPAR*-γ mRNA expression levels were found to be elevated in fish oil-fed animals.[26,27] However, it has remained unclear whether fish consumption mediates effects on CRC development through interactions with *PPAR*-γ. We also investigated potential interactions between the two polymorphisms of the *PPAR*-γ gene and fish consumption with regard to CRC risk.

Methods

Subject selection and data collection

The case-control study was conducted with 301 colorectal cancer patients and 291 controls. All subjects were residents of Chennai and the surrounding area in south-eastern India. Cases were recruited between 1999 and 2001 at the Madras Cancer Institute in Chennai, India, with all patients with a first diagnosis of histologically confirmed colorectal cancer being enrolled. Control subjects were cancer-free individuals, selected among visitors who were attending with patients admitted for having cancers other than CRC during the time period of case collection. They were frequency matched to case patients by sex and age (within 5 years). Informed consent was obtained from all study subjects. Trained interviewers collected information on the socioeconomic status, medical histories, alcohol drinking habit, and smoking and tobacco-chewing habits using a standard questionnaire. A 114 food and beverage item food-frequency questionnaire (FFQ) specific to this population was used to measure long-term intake of foods and food groups. Interviewers asked the subjects about the average frequency of consumption of food items per week over the past 1-year period (for cancer cases, this was 1 year before the diagnosis of CRC). Foods and food groups were categorized as follows: cereals and breads ($n = 11$ food items), beans ($n = 6$), vegetables ($n = 22$), meats ($n = 4$, including mutton, beef, pork and chicken), fish ($n = 7$, including river fish, sea fish and shellfish), fruit ($n = 13$), dairy products and eggs ($n = 10$), beverages ($n = 6$), snacks and desserts ($n = 18$), spices ($n = 7$) and oil ($n = 10$). After the interview, 7 mL blood from each fasting subject was collected and stored at −80°C. The internal review board of the Madras Cancer Institute in Chennai approved the study.

Genotyping

DNA samples of subjects were extracted from peripheral blood leukocytes. To assess *PPAR*-γ genotypes, we used polymerase chain reaction to amplify the regions of the *PPAR*-γ gene that contain the Pro12Ala substitution and the C161T transition.[3,18] A 270-bp fragment including Pro12Ala was amplified using forward primers (5′-GCCAATTCAAGCCCAGTC-3′) and reverse primers (5′-GATATGTTTGCAGACAGTGTATCAGTGAAGGAATCGCTTTCCG-3′), the Pro12Ala change creating a restriction site for the BstU-I enzyme. The expected products after digestion with BstU-I were 270 bp for Pro/Pro, 227 and 43 bp for Ala/Ala, and 270, 227 and 43 bp for Pro/Ala. A 200-bp fragment of C161T was amplified using forward and reverse primers (5′-CAAGACAACCTGCTACAAGC-3′ and 5′-TCCTTGTAGATCTCCTGCAG-3′, respectively), then digested with the Pml*I* restriction endonuclease. This resulted in two fragments (120 bp and 80 bp) for the wild type and one fragment (200 bp) when the restriction site was eliminated by the C161T transition. For quality control purposes, negative and positive controls were processed with each batch of samples. In addition, 10% of the subjects had their samples rerun to ensure agreement with the initial results.

Statistical analysis

We investigated the relationship between *PPAR*-γ genotypes and risk of CRC with the STATA statistical package (version 8.0; Stata Corporation, College Station, TX, USA). Differences of characteristics between cases and controls were assessed using the χ^2-test, as well as disparities of genotype and allele frequencies between the two groups. The Hardy–Weinberg equilibrium was checked using the χ^2-test. Unconditional logistic regression analysis was employed to estimate the odds ratios (OR) and confidence intervals (95% CI) for the association between genotypes and risk of CRC. Adjustments were made for matching variables (age, sex) and for possible confounders. Covariates were identified as potential confounders by examining their distribution by case-control status. As body mass index (BMI) in some cases were affected by the cancer, BMI was excluded from covariates to avoid information bias. The covariates were included in the model if they changed the OR by more than 20% or significantly changed the likelihood ratio statistic ($P < 0.05$) on univariate analysis. For all associations of genotypes with CRC, those subjects who were homozygous for the wild-type allele served as a reference. To increase statistical power, rare homozygotes were combined with heterozygotes assuming a dominant effect as their risk estimates were similar. To estimate linkage disequilibrium between *PPAR*-γ variants, pairwise linkage disequilibrium coefficients (D′) were calculated with the LINKAGE program.[28] The 'hapipf' command within STATA, which uses the expectation-maximization algorithm to resolve phase combined with a log-linear model, was used to estimate haplotype frequencies.[29] The χ^2-test was used to compare the distribution of haplotypes between cases and controls. The likelihood ratio test was used to examine the interaction among variables with respect to the risk of CRC. All statistical tests were two-sided and differences were considered to be statistically significant at $P < 0.05$.

Results

Demographic and lifestyle characteristics for the 301 colorectal cancer and 291 control subjects are shown in Table 1. In general, the CRC cases had a smaller BMI, a lower family income, and a higher prevalence of family history of CRC, and smoked more tobacco than the controls. In our population, after adjustment for sex, age, smoking habit, family history and family income, consumption of vegetables and fruit yielded a significant reduction of CRC risk ($P_{trend} = 0.001$ for vegetable intake, and $P_{trend} = 0.01$ for fruit intake). Fish intake

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Table 1. Characteristics of the colorectal cancer (CRC) patients and control subjects

	Cases (%) (n = 301)	Controls (%) (n = 291)	$P^{\dagger}$
Male	196 (65.1)	183 (62.9)	NS
Age (years)			
20–44	107 (35.6)	111 (38.1)	NS
45–59	109 (36.2)	121 (41.6)	
60–75	85 (28.2)	59 (20.3)	
BMI (kg/m^2)			
< 20.0	153 (50.8)	109 (37.5)	< 0.01
20.0–24.9	110 (36.6)	111 (38.1)	
≥ 25.0	38 (12.6)	71 (24.4)	
Education (years)			
< 5	104 (34.5)	88 (30.2)	NS
5–11	155 (51.5)	163 (56.0)	
> 11	42 (14.0)	40 (13.8)	
Religion			
Hindu	265 (88.0)	256 (88.0)	NS
Muslim	23 (7.7)	27 (9.3)	
Christian	13 (4.3)	8 (2.7)	
Family income (rupees/week)			
< 500	143 (47.5)	97 (33.3)	< 0.05
501–1300	69 (22.9)	101 (34.7)	
> 1300	89 (29.6)	93 (32.0)	
Smoking habit (pack-years)			
0	240 (79.7)	227 (78.0)	< 0.01
≤ 10	41 (13.6)	58 (19.9)	
> 10	20 (6.7)	6 (2.1)	
Drinking habit	56 (18.6)	56 (19.2)	NS
Tobacco chewing habit	39 (13.0)	28 (9.6)	NS
Family history of CRC	4 (1.3)	0	< 0.05
Vegetable intake (servings/day)			
< 2	117 (38.9)	65 (22.3)	< 0.01
2–3	109 (36.2)	111 (38.2)	
> 3	75 (24.9)	115 (39.5)	
Fruit intake (servings/week)			
< 4	132 (43.8)	102 (35.1)	< 0.05
4–8	126 (41.9)	129 (44.3)	
> 8	43 (14.3)	60 (20.6)	
Meat intake (servings/week)			
< 2	236 (78.4)	237 (81.4)	NS
≥ 2	65 (21.6)	54 (18.6)	
Fish intake (servings/week)			
< 2	251 (83.4)	219 (75.3)	< 0.05
≥ 2	50 (16.6)	72 (24.7)	

†Examined using the χ^2-test. BMI, body mass index; NS, not significant.

was related to a decreased risk of 0.63 (95% CI: 0.42–0.95), when comparing subjects who consumed two servings per week with those consuming less than two servings per week. In contrast, high meat intake (two servings per week) relative to low meat intake (less than two servings per week) conferred an increased risk (OR = 1.45, 95% CI: 0.92–2.35).

The genotype frequencies and association between the two polymorphisms and risk of CRC are shown in Table 2. The distribution of the observed genotypes did not deviate from the Hardy–Weinberg equilibrium for either the Pro12Ala (P = 0.77 in cases, and P = 0.83 in controls) or C161T (P = 0.33 in cases, and P = 0.71 in controls) polymorphisms. For the Pro12Ala polymorphism, the Pro/Pro, Pro/Ala and Ala/Ala genotype frequencies were 79.7%, 18.9%, and 1.3%, respectively, in the cancer cases compared with 79.0%, 19.6%, and 1.4%, respectively, for the controls. For the C161T polymorphism, the C/C, C/T and T/T genotype frequencies were 69.8%, 26.6% and 3.6%, respectively, in the cancer cases compared with 76.0%, 22.7% and 1.3%, respectively, for the controls. No significant differences in the genotype distribution of the Pro12Ala and C161T polymorphisms were observed between the cases and controls (P = 0.98 and P = 0.09). The T allele frequency for the C161T polymorphism was greater among cancer patients than controls (0.169 vs 0.127, P = 0.04), but no difference in the Ala allele frequency with the Pro12Ala polymorphism was found (0.108 vs 0.112).

After adjustment for sex, age, smoking habit, family history and family income, the OR was 1.52 (95% CI: 1.02–2.25) for the C/T genotype, and 2.71 (95% CI: 0.82–8.99) for the T/T genotype compared to the C/C genotype with the C161T polymorphism. When the C/T genotype and T/T genotypes were grouped, the OR was 1.61 (95% CI: 1.10–2.36). This association was essentially the same when colon and rectal cancers were analyzed separately. Compared to the Pro/Pro genotype, the OR was 1.07 (95% CI: 0.70–1.63) for the Pro/Ala genotype, and 1.02 (95% CI: 0.25–4.28) for the Ala/Ala genotype. When the Pro/Ala and Ala/Ala genotypes were grouped, the OR was 1.06 (95% CI: 0.70–1.61). Calculations based on the prevalence of the two polymorphisms and the size of our study population suggested an 80% power to detect an association at the 5% significance level (two-sided test) if the Pro12Ala and C161T polymorphisms conferred at least a two-fold increased risk (carriers of at least one variant allele vs no variant allele).

The haplotype frequency was computed from genotype data and the results are presented in Table 3. Linkage disequilibrium between Pro12Ala and C161T polymorphisms was observed ($D' = 0.69$, $\chi^2 = 234$ and $P < 0.001$ in controls; $D' = 0.88$, $\chi^2 = 282$ and $P < 0.001$ in cancer cases). A significant difference in haplotype frequencies between cancer cases and controls was found ($\chi^2 = 11.62$, $P = 0.009$, d.f. = 3). The frequency of the Pro-T haplotype (Pro allele for Pro12Ala and T allele for C161T) was higher in cancer cases than in controls (7.6 vs 3.9%). In contrast, the frequency of the Ala-C haplotype was lower (1.1% vs 2.7%).

Table 4 presents data for associations between the two polymorphisms in the *PPAR*-γ gene and CRC risk stratified for fish intake. A significant association between the C/T + T/T genotype in the C161T polymorphism and CRC risk limited to the subgroup of those who had a low fish intake was found. For the C/T + T/T genotype, high fish intake decreased the risk from 1.85 (95% CI: 1.20–2.89) to 0.69 (95% CI: 0.32–1.50). The P-value for the interaction was 0.10. There were no significant interactions between fish intake and the Pro12Ala polymorphism with regard to CRC risk.

Discussion

The present investigation, conducted to explore associations between the Pro12Ala and C161T polymorphisms in the *PPAR*-γ gene and CRC in an Indian population, showed the C/T + T/T genotype to be associated with a significant 1.61-fold increase in the OR compared with the C/C genotype with the C161T polymorphism. Analysis of the Pro-T haplotype

Table 2. Odds ratios (OR) for colorectal cancer (CRC) with reference to the *PPAR*-γ genetic polymorphisms

Variable	All cases		Colon cancer		Rectal cancer		Controls
	n	OR† (95% CI)	*n*	OR† (95% CI)	*n*	OR† (95% CI)	*n*
Pro12Ala							
Pro/Pro	240	1.00 (reference)	46	1.00 (reference)	194	1.00 (reference)	230
Pro/Ala	57	1.07 (0.70–1.63)	13	1.36 (0.66–2.78)	44	0.99 (0.63–1.57)	57
Ala/Ala	4	1.02 (0.25–4.28)	0	NA	4	1.27 (0.30–5.36)	4
Pro12Ala (grouped)							
Pro/Pro	240	1.00 (reference)	46	1.00 (reference)	194	1.00 (reference)	230
Pro/Ala + Ala/Ala	61	1.06 (0.70–1.61)	13	1.20 (0.59–2.43)	48	1.01 (0.65–1.58)	61
C161T							
C/C	210	1.00 (reference)	37	1.00 (reference)	173	1.00 (reference)	221
C/T	80	1.52 (1.02–2.25)	19	1.95 (0.99–3.81)	61	1.42 (0.96–2.18)	66
T/T	11	2.71 (0.82–8.99)	3	3.09 (0.53–18.06)	8	2.61 (0.75–9.07)	4
C161T (grouped)							
C/C	210	1.00 (reference)	37	1.00 (reference)	173	1.00 (reference)	221
C/T + T/T	91	1.61 (1.10–2.36)	22	2.00 (1.05–3.81)	69	1.50 (1.01–2.26)	70

†Adjusted for sex, age, smoking habit, family history and family income. CI, confidence interval; NA, not available.

Table 3. Haplotype frequencies for the *PPAR*-γ gene in the colorectal cancer patients and control subjects

	Frequency among cases	Frequency among controls	*P*§
PPAR-γ haplotype†			
Pro-C	0.816	0.849	0.009
Pro-T	0.076	0.039	
Ala-C	0.011	0.027	
Ala-T	0.097	0.085	
Disequilibrium			
D′‡	0.881	0.686	
χ^2	282	234	
P	0.0001	0.0001	

†The order of single-nucleotide polymorphisms in the haplotypes is Pro12Ala-C161T. ‡Pair-wise linkage disequilibrium coefficients. §The χ^2-test was used to compare the distribution of *PPAR*-γ haplotypes between cases and controls.

strengthened the relationship in our study population, and this proved consistent for both the colon and rectum.

However, evidence concerning the relationship between the C61T polymorphism and cancer is still limited and controversial. A protective effect on colorectal adenomas was earlier found for the C/T genotype of the C161T polymorphisms in *PPAR*-γ,[21] but other studies have shown an increased risk.[24,25] Clearly, functional aspects require further assessment.

Three hypotheses may be proposed for how CRC might be affected by the polymorphisms examined here. First, a new cryptic splice donor, acceptor or enhancer may be created by this C/T substitution, with decreased expression of the variant bearing the T allele, thus leading to a low level of functional activity. Alternatively, the substitution may influence the stability of the mRNA species. Second, it is reported that the T allele of the C161T polymorphism is associated with elevated plasma levels of leptin,[30] a 16 kDa adipokine that regulates proinflammatory immune responses,[31] and may be a growth factor for colonic epithelial cells.[32] Case-control studies have in fact suggested that leptin is a risk factor for colorectal cancer.[33,34] Third, C161T polymorphism may be in linkage disequilibrium with functional mutations in other *PPAR*-γ gene exons, or other unidentified genes near the *PPAR*-γ gene. Controversial results have been obtained for the associations between the C161T polymorphism and risk of colorectal adenomas.[21] Reasons for disagreements may be due in part to differences in study populations.

Analysis with estimated haplotypes showed the Pro-T haplotype to be more prevalent in cancer cases than in controls (7.6% vs 3.9%). Although relatively uncommon because

Table 4. Odds ratios for interactions between *PPAR*-γ genotypes and colorectal cancer stratified by fish intake

	Low fish intake†		*P*	High fish intake†	
	Cases/controls (*n*)	OR‡ (95% CI)		Cases/controls (*n*)	OR‡ (95% CI)
Pro12Ala					
Pro/Pro	197/176	1.00 (reference)		43/54	0.74 (0.46–1.18)
Pro/Ala + Ala/Ala	54/43	1.14 (0.72–1.81)		7/18	0.51 (0.20–1.27)
Interaction			0.36		
C161T					
C/C	171/173	1.00 (reference)		39/48	0.83 (0.51–1.36)
C/T + T/T	80/46	1.85 (1.20–2.89)		11/24	0.69 (0.32–1.50)
Interaction			0.10		

†Low fish intake, less than two servings per week; high fish intake, greater than two servings per week. ‡Adjusted for sex, age, smoking habit, family history, family income and consumption of meat, vegetables and fruit. CI, confidence interval; OR, odds ratio.

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of linkage disequilibrium between the two polymorphisms, its importance was also reported in two other studies regarding bodyweight and colorectal adenomas.[21,35] The validity of haplotype inference varied, depending on a number of factors, including sampling error, sample size, number of loci studied, allele frequencies, locus-specific allelic departures from the Hardy–Weinberg equilibrium and the linkage-disequilibrium structure of the region.[36] In the present study, to prevent genotyping bias, negative and positive controls were processed with each batch of samples, and 10% of the subjects had their samples rerun to ensure agreement with the initial results. Haplotype block predictions were based on all the complete genotype data available for the cases and controls with use of the expectation-maximization algorithm. In addition, the state of sample size (cases = 301, controls = 291), number of loci studied (two loci), allele frequencies (minor allele of the two polymorphisms < 0.17), Hardy–Weinberg equilibrium ($P = 0.83$ for Pro12Ala and $P = 0.71$ for C161T in controls; $P = 0.77$ for Pro12Ala and $P = 0.33$ for C161T in cancer cases), and linkage disequilibrium ($D' = 0.69$ in controls, and $D' = 0.88$ in cancer cases) in our study also supported the validity of haplotype estimation.[36] Certainly, more detailed investigations will be necessary to allow accurate haplotype inferences in future.

The Pro12Ala polymorphism in the *PPAR-γ* gene has been investigated in breast, prostate, lung and endometrial cancers and colorectal adenomas, but no significant associations were detected.[21–23,37,38] Landi *et al*. have reported the Ala allele to be related to a reduced risk of CRC in a hospital-based case-control study.[19] We could not confirm this finding in our study population. Tomita *et al*. have reported that the Pro12Ala polymorphism might be implicated in development of CRC in which the K-*ras* gene is not mutated.[39] Variation in lifestyle patterns or genetic background among the Indian and Spanish populations may explain to some extent the observed differences in risk. Our study used population-based controls, whereas Landi *et al*. adopted hospital controls, among which the Ala allele frequency was greater than in the Spanish general populace (0.11 vs 0.09).[40] In present study, the frequency of the Ala allele was similar to that in the general population in a diabetes study of Singapore Indians (0.112 vs 0.119).[41]

Epidemiological studies have shown that fish consumption is protective against CRC,[42] and the risk-reducing effects were also found in the present study. However, there were no significant interactions between the two polymorphisms and fish consumption in CRC development. Because only 20% of the study subjects consumed more than two servings of fish per week, this combined with the low allele frequency of the two polymorphisms may account for the non-significant results. In addition, as exposure information was collected after the diagnosis of CRC, differential dietary recall between cases and controls could yield biased results. Further larger studies in future are clearly warranted. While it is known that two polymorphisms in the *PPAR-γ* gene can affect the susceptibility to diabetes,[2,18] we failed to collect data on this disease in the present study.

In conclusion, our investigation here indicated that the *PPAR-γ* gene C161T substitution might be associated with an increased CRC risk, but no significant link was observed for the Pro12Ala polymorphism. As little is known about the underlying physiology, further genetic and epidemiological studies of the *PPAR-γ* gene loci and other associated genes should be conducted with an emphasis on functional aspects.

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