Relatıon between common polymorphisms in genes related to inflammatory response and colorectal cancer

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Abstract

AIM: To investigate the association between common single nucleotide polymorphisms (SNPs) in inflammatory response-related genes such as interleukin (IL)-6, IL-8, tumor necrosis factor α (TNFα), peroxisome proliferators-activated receptor γ (PPARγ), intercellular adhesion molecule-1 (ICAM-1) and the risk of colorectal cancer (CRC) in a group of Greek patients.

METHODS: The study group consisted of 222 CRC patients and 200 healthy controls. Genotyping was performed using allele-specific PCR of PRC-RFLP and the results were confirmed by sequencing. We studied the association of SNPs in the IL-6 (-174G > C), IL-8 (-251T > A), TNFα (-308G > A), ICAM-1 (R241G and K469E), and PPARγ (Pro12Ala) genes and the risk of CRC.

RESULTS: The IL-6 -174G, R241 and K469 alleles of ICAM-1 were associated with increased risk of CRC (OR = 1.77, 95% CI: 1.34-2.34; OR = 1.83, 95% CI: 1.23-2.72; and OR = 1.35, 95% CI: 1.03-1.77 respectively). The IL-8 and TNFα polymorphisms had no effect. Whereas the PPARγ Pro12 genotype was associated with increased risk of disease (OR = 1.78, 95% CI: 1.25-2.49).

CONCLUSION: The association between common SNPs in immunologic response-related genes and CRC is reported in the present study. Apart from shedding light on the mechanisms of malignancy initiation and progression, SNPs may improve appropriate screening for sub-populations at risk.

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Key words: Colorectal cancer; Inflammation; Single nucleotide polymorphisms

INTRODUCTION

Colorectal cancer (CRC), whether sporadic or hereditary, is caused by a defined set of molecular events[1]. A wealth of knowledge has been acquired about the precise molecular events driving CRC formation. Germline mutations in tumor-suppressor adenomatous polyposis coli (APC) genes and DNA mismatch repair (MMR) genes lead to the recognized familial adenomatous polyposis (FAP)-related CRC and the hereditary non-polyposis colorectal cancer (HNPPC), respectively[2]. These inherited cases account for about 5%-10% of CRC[3]. Hereditary factors may then contribute to an estimated further 20% of CRC, especially in patients with a strong family history of CRC[4]. So, up to a quarter of all CRC may occur due to some form of inherited susceptibility to CRC, either in the context of the recognized clinical syndrome or due to germline variants which may carry an increased risk of CRC[5]. Even without a family history of CRC, 1 in 21 of the population develops the disease, usually later in life[6]. Sporadic cases of CRC result from somatic hits to the aforementioned genes[7]. Germline mutation detection is not capable of predicting and screening for sporadic CRC. In other words, excluding inherited types of CRC, the susceptibility of a certain individual to development of sporadic CRC remains largely undetermined.

Since most CRCs arise sporadically, environmental and host immunological factors could significantly
contribute to the initiation and even the progression of this malignancy. Indeed, colonic cells respond to various malignancies-contributing environmental factors based on the genotype of DNA loci associated with metabolic pathways that relate to various dietary constituents. Genetic polymorphisms are thought to play a role in determining how individuals respond at the cellular level to various environmental factors.

Immune response undoubtedly has a significant impact on the potential for malignancy, which is highlighted by the close association between chronic inflammatory conditions and subsequent malignant transformation in the inflamed tissue. Inflammation favors tumorigenesis by stimulating angiogenesis, damaging DNA and chronically stimulating cell proliferation. The mixture of cytokines that is produced in the tumor microenvironment plays an important role in cancer pathogenesis. Cancer cells can also respond to host-derived cytokines that promote growth, attenuate apoptosis and facilitate invasion and metastasis. Importantly, cytokine genetic polymorphisms have recently emerged as determinants of gastrointestinal malignant disease susceptibility and severity. Although rather limited, evidence already exists linking common polymorphisms in immunologic parameters to CRC tumorigenesis.

Bacterial flora keeps the normal colon mucosa in a continuous state of low-grade inflammation, stimulating release of various pro-inflammatory cytokines by the immune cells. Cytokines activate the NF-κB transcription factor signal pathway in epithelial and immune cells, leading to up-regulation of interleukin-6 (IL-6) and IL-8. IL-6 and IL-8 have pro-inflammatory activity in the intestine via the STAT3 intracellular signal pathway. IL-6 also acts as a potent stimulator of metastasis by up-regulating the expression of adhesion receptors on endothelial cells, such as the intercellular adhesion molecule-1 (ICAM-1). The status of a common functional single G > C base exchange in the human IL-6 gene promoter (chromosome 7p21) increases IL-6 levels. It has been found that the A-allele of the -251 T > A SNP in the IL-8 gene is related to higher IL-8 levels after stimulation with lipopolysaccharide and respiratory syncytial bronchiolitis in children. Tumor necrosis factor α (TNFα) is a pro-inflammatory cytokine whose role has been established in the pathogenesis of rheumatoid arthritis and inflammatory bowel disease (IBD).

This study focused on the investigation of the aforementioned SNPs as host risk factors for sporadic CRC, using a Greek population cohort.

**MATERIALS AND METHODS**

**Subjects**

Two hundred and twenty-two consecutive Greek patients with CRC without previous diagnosis of IBD or any of the known hereditary cancer syndromes (128 males, mean age at diagnosis 66.21 ± 10.67 years and 94 females, mean age at diagnosis 63.12 ± 11.52 years) and 200 healthy sex- and age-matched controls (120 males, mean age 64.73 ± 15.32 years and 80 females, mean age 60.58 ± 12.35 years) were genotyped. The vast majority of the studied group consisted of truly sporadic cases (198 patients, 89.2%), while 24 patients (10.8%) reported CRC occurrence in at least one first degree relative. Overall, 35.1% of CRC patients had a family history of malignancy.

**Genotypic analysis**

DNA was extracted from peripheral blood using the QIAamp blood kit (Qiagen, Germany). To confirm the integrity of DNA, a 430-bp sequence in the human glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) gene was amplified.

The IL-6 polymorphism at position 174 was analyzed by allele specific PCR as previously described. Briefly, we used primers framing a 347-bp region surrounding the G-174C allele to amplify the genomic DNA isolated from patients and controls by PCR (sense, 5'-TTGTCA AGACATGCAAGTGC-3'; anti-sense, 5'-CAGAATG AGCCTCAGACATCTCC-3'). In addition, each PCR reaction contained two additional primers designed to detect the G-174C allele (anti-sense, 5'-GCAATGACGT CCTTATGACTG-3') and another primer designed to detect the G-174C allele (sense, 5'-CCCCCTAGTTG TCTTGCCA-3'). We performed multiplex PCR with all the four primers in one tube. PCR products were isolated on 3% agarose gels and visualized with ethidium bromide staining. Individuals with the IL-6-174 C/C genotype were considered low producers and those with the IL-6-174 G/C or G/G genotype were considered high producers.

Several immunologic disorders, including inflammatory bowel disease are associated with distinct polymorphisms of ICAM-1.

Peroxisome proliferators-activated receptor γ (PPARγ) is a ligand-activated nuclear transcription factor, which plays a central role in orchestrating gene expression in response to exogenous ligands like other PPARs, such as non-steroidal anti-inflammatory agents (NSAIDs). Its implication in immunologic mechanisms and carcinogenesis has been strongly speculated. Natural ligands and drug agonists of PPARγ reduce intestinal inflammation in IBD patients via inhibition of the NF-κB and STAT3 pathways, while inactivating PPARγ mutations have been found in sporadic CRC. PPARγ has a polymorphism in the coding region 34C > G that results in the aminoacid change of Pro12Ala.

This study focused on the investigation of the aforementioned SNPs as host risk factors for sporadic CRC, using a Greek population cohort.
Allele specific primers were 5’-CCACAATTTGGTGAATTATCAAT-3’ (-251A) or 5’-CCACAATTTGGTGAATTATCAAT-3’ (-251T). The consensus primer was 5’-TGCCCCCTCAGGCTTAA-3’, giving a PCR product of 336 bp. PCR products were isolated on 2% agarose gels and visualized with ethidium bromide staining.

The A-allele of a single nucleotide polymorphism in the promoter region of the TNFα gene (-308G > A) was determined by PCR-RFLP. The primer sequences used were: forward 5’-AGGCAATAGGTTTGGAGGCCT-3’ and reverse 5’-TCCTCGGCTCCGATTCCGA-3’, which could amplify a 107 bp sequence. PCR products with a G at position -308 were digested by Nco I to give two fragments of 87 bp and 20 bp. Those with an A-allele at position -308 were not digested by Nco I as previously described[28].

Single base polymorphism at codon 241 (R241G) in exon 4 was determined using allele specific PCR as previously described[29]. We used the primers (5’-GGGTTGGTAGTGTCATTTTGGA-3’ and 5’-GCTGCTGCTGCTGAGCAGC-3’) with the last nucleotide complementary to the allelic variant substitution base on the point mutation in question of the gene ICAM-1, and a common primer (5’-GGCTCAGCAGCGTGA-3’). The amplified PCR products of 137 bp were analyzed by 2% agarose gel electrophoresis followed by ethidium bromide and ultraviolet visualization. The second amino acid polymorphism at codon 241 (R241G) was confirmed by sequencing ready reaction kit (Applied Biosystems), and sequencing analysis using a dye terminator cycle sequencing ready reaction kit (Applied Biosystems). As negative control of sequencing reactions, we used distilled water instead of genomic DNA and confirmed the fidelity of the reactions.

Exon 2 of PPARγ was amplified using PCR and the primers G2F (5’-CTGATGTCTTGACTTGAGGGA-3’) and G2R (5’-GGAGCAAAAATCATACAAGAGC-3’) as previously described[30]. The 295 bp PCR product was digested overnight with HpaI, which cleaves the Ala allele to generate DNA fragments 178 and 117 bp in size. The DNA fragments were separated on 3% agarose gel.

In all cases, the mutations were confirmed by sequencing analysis using a dye terminator cycle sequencing ready reaction kit (Applied Biosystems), and an ABI 377 automated sequencer. As negative control of the PCR amplifications, we used distilled water instead of genomic DNA and confirmed the fidelity of the reactions.

Statistical analysis

Frequency and susceptibilities of mutations among CRC patients and controls were compared with the chi-square ($\chi^2$) distribution test. Odds ratios (OR) for association were estimated using logistical regression accounting for possible covariates: age, sex and for influence by the remaining genotype loci. Hardy-Weinberg equilibrium for the genotypes was tested using the $\chi^2$ distribution test for the difference in the observed and expected frequencies. All tests were 2-tailed and $P < 0.05$ was considered statistically significant. Inference was aided by SPSS (version 11.0.1, SPSS Inc., Chicago, IL).

Table 1 Tumor parameters in colorectal cancer patients

| Parameters | Patients (n) |
|------------|-------------|
| Tumor location | Rectum 70, Left colon 104, Right colon 48 |
| Tumor size | ≤ 3 cm 69, > 3 cm 153 |
| Growth pattern | Ulcerative 89, Protruding 133 |
| Differentiation | Good 43, Moderate 147, Poor 32 |
| TNM stage | I 32, II 96, III 68, IV 26 |

RESULTS

Tumor characteristics are depicted in Table 1. Among the CRC patients, the distribution of the IL-6 genotypes was as follows: GG in 111 patients (50%), CG in 76 patients (34.23%) and CC in 35 patients (15.76%). The distribution of genotypes differed significantly from that in the healthy individuals (Table 2). Additionally, the allele IL-6 -174G was associated with increased risk of CRC, since it was found to be overrepresented among CRC patients (67.1% vs 53.5%, $P < 0.0001$, $\chi^2 = 16.35, OR = 1.77, 95\% CI: 1.34-2.34$).

Concerning the A allele and AA genotype frequencies of IL-8 at position -251, no significant differences were observed between CRC patients and healthy controls (Table 2). Similarly, no effect of the TNFα (-308G > A) polymorphism was found (Table 2).

Table 2 also summarizes the allelic frequencies and genotypes of the ICAM-1 gene in CRC patient group and controls. No significant differences were observed in the RR (for the R241G) or in the KK (for the K469E) genotypes of ICAM-1 gene in CRC patient group and controls. Nevertheless, the allelic frequency of both R241 and K469 was significantly higher in CRC patients than in controls (P = 0.002, $\chi^2 = 9.24$, OR = 1.83, 95% CI: 1.23-2.72; and P = 0.031, $\chi^2 = 6.02$, OR = 1.35, 95% CI: 1.03-1.77, respectively).

As also indicated in Table 2, the C allele (Pro12) of PPARγ was significantly associated with an increased risk of CRC since it was found to be overrepresented among CRC patients compared to controls (84.7% vs 76.5% respectively; P = 0.001, $\chi^2 = 9.08$, OR = 1.78, 95% CI: 1.25-2.49). Consequently, the CC genotype appeared as the major risk genotype for CRC ($P < 0.001$, $\chi^2 = 16.43$, OR = 2.01, 95% CI: 1.32-3.09).

When co-carriage rates of the offending alleles (IL-6 -174G, ICAM-1 R241, ICAM-1 K469) were compared...
DISCUSSION

The most compelling evidence for the role of inflammation in gastrointestinal (GI) malignancy comes from studies showing that pro-inflammatory cytokine gene SNPs increase the risk of cancer and its precursors. A number of pro-inflammatory genotypes related to known cytokines (IL-1β, TNFα, IL-10) increase the risk of *H pylori*-induced gastric atrophy and gastric cancer. At present, relatively limited information exists on the relationship between colorectal malignancies and cytokine polymorphisms. Polymorphisms in the IL-6 gene promoter and the R241 and K469 ICAM-1 polymorphic sites were associated with a significantly increased risk of CRC, whereas the PPARγ CC genotype and C allele were both related to reduced risk in the present study.

Our results contrast with the findings of Landi et al in terms of the influence of IL-6 SNPs on CRC predisposition, but parallel their results as far as the potential protective role of the PPARγ Ala12 variant. The researchers concluded that the IL-6-174 C genotype confers an increased risk of CRC, but in our study, a similar outcome was related to the presence of the G allele in the respective gene. The biological role of the substitution 174G > C has not been fully elucidated by functional studies. Although the C-174 allele is associated with both lower and higher levels of IL-6 expression in various conditions, one study has been conducted to evaluate the effect of -174G > C SNP on IL-6 serum levels in CRC patients, and found that the GG genotype and absence of the C allele are related to significantly increased levels of IL-6, particularly in the presence of hepatic metastasis. Accordingly, in vitro, macrophages from C-negative subjects produce higher IL-6 levels than those from C-positive subjects. Moreover, the fact that high levels of circulating IL-6 are observed in patients with different tumor types and the finding of different levels of IL-6 expression in relation to genetic variants suggest that this cytokine is mainly produced by host cells rather than by cancer cells. We could speculate that the IL-6 G allelic variant might influence the increased cytokine production by immune cells, which in turn induces its stimulatory effect in tumorigenesis. Interestingly as well, Landi et al reported that the C genotype is associated with an increased risk of CRC only in those subjects who do not habitually take NSAIDs. A possible cause for the conflicts and mismatches, like those observed here and the earlier study in allele and genotype distributions, may be the differences in ethnic backgrounds. The IL-6-174 C allele’s protective role against CRC, as having been demonstrated by Landi et al could not be replicated in our study. It is true that the function of this polymorphism and the role of IL-8 in intestinal inflammation have not yet been.
adequately studied\textsuperscript{[19]}. Previous reports support that there is no significant association between the TNF\(\alpha\) -308A allele and CRC development like in our study\textsuperscript{[3,39]}. The latter possibly precludes any hypothetical involvement of TNF\(\alpha\)-308A in CRC tumorigenesis. In contrast to the aforementioned lack of relevance, the examined TNF\(\alpha\) SNP has been found to participate in a genetic profile associated with a high risk for gastric cancer\textsuperscript{[12,49]}. The pro-inflammatory and acid inhibiting properties of TNF\(\alpha\) seem to enhance \(H.\) pylori oncogenic or other effects on gastric mucosa\textsuperscript{[12,44]}. Although a similar extrapolation regarding certain large bowel–colonizing bacteria could be made, such a hypothesis would not be supported based at least on our results. Recent studies on SNPs at the TNF\(\alpha\)-308 locus have not found any difference between prostate and breast cancer patients or controls\textsuperscript{[41,42]}. In contrast and at least for breast cancer, assays for germline SNPs in TNF\(\beta\) instead of TNF\(\alpha\) may prove useful, since at least for breast cancer, the TNF\(\beta\) G/G genotype increases the risk of tumorigenesis, while the A allele inhibits that risk\textsuperscript{[42]}. The present study is the first study investigating ICAM-1 SNPs in CRC and reporting the increased allelic frequency of R241 and K469 polymorphisms in this cancer. Previous studies also reported that both R and K allelic variants are associated with IBD\textsuperscript{[16,19,30]}, which adds to the mounting molecular evidence about the similarities between IBD-associated cancer and sporadic cancer\textsuperscript{[46]}. Based on ample data concerning the molecular mechanisms involved in colonic mucosal inflammation effects, it is reasonable to speculate that even sporadic cancer might be largely secondary to inflammation\textsuperscript{[34]}. The normal colon could be viewed as being in a perpetual state of inflammation, where cytokine profiles, glycosylation changes, other inflammatory molecule (i.e. prostaglandins) regulation and mucosa-associated bacteria play a central role\textsuperscript{[38]}. Adhesion molecule activity may also have a serious impact on immunologic response. R241 and K469 amino acid changes in the ICAM-1 gene may influence its functional role, as both are located on the Mac-1 binding domain and in the immunoglobin-like domain 5, respectively. The relevance of these specific SNPs could be explained by the functional alteration of the gene product\textsuperscript{[39]}. Some of the inhibitors of HMG-CoA reductase (statins) have also been shown to bind to the ICAM-1 binding domain of LFA-1\textsuperscript{[43]}. The regulation of interaction mediated by adhesion molecules may provide a new target for controlling inflammatory and immune responses.

In keeping with Landi \textit{et al}\textsuperscript{[39]}, the PPAR\(\gamma\) Ala12 variant is associated with reduced CRC risk. In our study, the PPAR\(\gamma\) CC genotype (Pro12) had the strongest relative risk for CRC among the examined genes. The protective effect of the Ala12 variant of PPAR\(\gamma\) gene has been recently confirmed by Gong \textit{et al}\textsuperscript{[44]} in sporadic colorectal adenomas. Similarly the polymorphic allele Ala12 is significantly over-represented in glioblastoma multiform patients, but such an association could not be verified in prostate cancer\textsuperscript{[25,45]}. The PPAR\(\gamma\) Ala12 is associated with increased tissue sensitivity to insulin, a decrease in insulin plasma level and reduced release of free fatty acids by adipocytes, which in turn are associated with reduced CRC risk\textsuperscript{[46,47]}. Other plausible mechanisms accounting for this protective effect may lie in PPAR\(\gamma\) involvement in regulation and interactions with other colonic cell functions. Activation of PPAR\(\gamma\) by ligands inhibits the NF-\(\kappa\)B and STAT3 pathways attenuating IBD and related conditions, induces differentiation and apoptosis of CRC cells and decreases DNA synthesis in CRC cell lines\textsuperscript{[23,46-50]}. The recently investigated transcriptional regulation of the tumor suppressor PTEN gene via PPAR\(\gamma\) may provide a mechanism for phosphatidylinositol 3-kinase (PI-3kinase)-mediated signaling cascades\textsuperscript{[51]}. Disruption of PPAR\(\gamma\) expression seems to prevent the up-regulation of PTEN leading to resistance of tumor cells (i.e. pancreatic cancer cells) and macrophages to apoptosis\textsuperscript{[52]}. The latter may open a new dimension in fully understanding the complex mechanisms involved in the interaction of PPAR\(\gamma\) and various tumorigenesis elements.

PPAR\(\gamma\) activation can lead to altered expression of COX-2, while NSAIDs have been reported to be capable of activating PPAR\(\gamma\)\textsuperscript{[53]}. NSAIDs can also alter synthesis of eicosanoids that may bind to and act as ligands for PPAR\(\gamma\) through their effects on COX activity\textsuperscript{[54,55]}. Acetylsalicylic acid causes an approximate halving of risk for sporadic CRC\textsuperscript{[56]}. In an era of ongoing attempts to identify suitable target population groups for chemoprevention with anti-inflammatory agents, such SNP analysis and mapping of suitable loci may prove invaluable.

It is noteworthy that other immunology-unrelated genes may also carry an increased risk of developing CRC. It was reported that the Harvey ras-1 variable number tandem repeat (HRAS1-VNTR) polymorphism, the methylenetetrahydrofolate reductase (MTHFR) valine/valine polymorphism and missense mutations in CDH1 (E-cadherin) gene all are associated with an increased risk of CRC tumorigenesis\textsuperscript{[35]}. Apart from shedding light on the mechanisms of sporadic malignancy initiation and progression, SNPs may provide appropriate screening recommendations in intermediate-risk patients, such as those with family history. Only a limited subset of our cohort consisted of such patients and no significant differences were derived in our analysis. Although rather difficult and possibly imprecise during the process of similar studies, taking relevant dietary and lifestyle habits into account is of paramount importance. Simultaneous genotyping and combined analysis of different SNPs in large numbers of patients and controls, stratified by ethnicity, gender and tumor location may make it possible to describe the exact relations between polymorphisms and CRC susceptibility with higher power and may open the way for population-wide genetic screening in the future.

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