The Inducible Prostaglandin Biosynthetic Enzyme, Cyclooxygenase 2, Is Not Mutated in Patients with Attenuated Adenomatous Polyposis Coli

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ABSTRACT

Germ-line mutations in the APC gene cause adenomatous polyposis coli (APC), a syndrome in which patients develop hundreds to thousands of precancerous adenomatous colorectal polyps. We described previously an attenuated form of APC (AAPC) resulting from very 5' mutations in APC in which affected patients exhibit fewer colorectal polyps and a later age of onset of colorectal cancer. However, because striking variations in colorectal polyp numbers occur among patients carrying identical AAPC mutations, alleles of another gene may modify the expression of the APC disease phenotype. We tested the hypothesis that loss of function of human cyclooxygenase 2 (COX-2), known to modify the APC phenotype in the Apc(5716) mouse, results in a decreased tumor burden in AAPC patients that develop very few colorectal polyps. Genomic DNA sequence analysis of human COX-2 revealed a silent mutation in exon 3 that was evenly distributed between two classes of patients with AAPC, those with small or large numbers of colorectal polyps. We also found no difference in levels of COX-2 mRNA in transformed blood lymphocytes among AAPC patients of either class or patients with classical APC, and no alterations that correlated with a lesser or greater number of colorectal polyps were detectable within approximately the first kb of the promoter sequence. Therefore, mutation of the human COX-2 gene does not appear to be responsible for a low tumor burden among AAPC subjects.

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An inherited predisposition to colon cancer, APC, is caused by germ-line mutations in the APC gene (1–4). Several mouse models exist that resemble human APC (5–7). In both humans and mice, the severity of the disease phenotype varies among individuals with identical APC mutations (8, 10). In humans, the variation in phenotype occurs both in the number of colorectal polyps that develop in affected individuals, including those with AAPC, and in the extent of extracolonic manifestations that develop in patients with Gardner syndrome (8). No genetic modifier of APC has been described in humans. However, in one of the murine models of APC, the Min mouse, variations in APC phenotype involving primarily tumor multiplicity and size, result from the effects of mutation in the gIIa secretory phospholipase A2 gene, Pla2g2a (9, 10).

COX-1 and COX-2 catalyze the committed step in the synthesis of prostaglandins (11). Several nonsteroidal anti-inflammatory drugs inhibit the activity of both forms of COX and reduce the occurrence and progression of colorectal polyps and cancer in humans and rodents (11–13). Administration of the nonsteroidal anti-inflammatory drug Sulindac can inhibit the number and size of colorectal polyps that develop in patients with APC or Gardner syndrome (14–17).

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COX-2 is not mutated in patients with attenuated APC.

Table 1 Primers used to amplify COX-2 DNA

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer 5'</th>
<th>Primer 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PHS21-F: 5'-UP-AGTCAATCCATGAGGAACTCTG-3'</td>
<td>PHS21-R: 5'-RP-TAAGGCAATACTAATCGG-3'</td>
</tr>
<tr>
<td>2</td>
<td>PHS22-F: 5'-UP-AGCTGGCTCTCCGCTGGAATG-3'</td>
<td>PHS22-R: 5'-RP-TGTTGAGCTTAGGCTG-3'</td>
</tr>
<tr>
<td>3</td>
<td>PHS23-F: 5'-UP-GTATAGCTTTAGGCTTATAG-3'</td>
<td>PHS23-R: 5'-RP-TAAGGCAATACTAATCGG-3'</td>
</tr>
<tr>
<td>4</td>
<td>PHS24-F: 5'-UP-GATTATAATCACTGAGGGAG-3'</td>
<td>PHS24-R: 5'-RP-TATTGAGCTTAGGCTG-3'</td>
</tr>
<tr>
<td>5</td>
<td>PHS25-F: 5'-UP-AGATGCTTGTCTAGGACAGT-3'</td>
<td>PHS25-R: 5'-RP-TGTTGAGCTTAGGCTG-3'</td>
</tr>
<tr>
<td>6</td>
<td>PHS26-F: 5'-UP-ATATGAGCTTGTCTAGGACAGT-3'</td>
<td>PHS26-R: 5'-RP-TAAGGCAATACTAATCGG-3'</td>
</tr>
<tr>
<td>7</td>
<td>PHS27-F: 5'-UP-AATGAGCTTGTCTAGGACAGT-3'</td>
<td>PHS27-R: 5'-RP-TAAGGCAATACTAATCGG-3'</td>
</tr>
<tr>
<td>8</td>
<td>PHS28-F: 5'-UP-CTTGAAAGCTTGTGATCAGATG-3'</td>
<td>PHS28-R: 5'-RP-TAAGGCAATACTAATCGG-3'</td>
</tr>
<tr>
<td>9</td>
<td>PHS29-F: 5'-UP-GTTTGAACATTGGTTGTATCTCTG-3'</td>
<td>PHS29-R: 5'-RP-AAGAATTCTTGGGAAATCTG-3'</td>
</tr>
<tr>
<td>10</td>
<td>PHS30A-F: 5'-UP-AGGTCAGAGCGGAACTCTG-3'</td>
<td>PHS30A-R: 5'-RP-AGTCAATCCATGAGGAACTCTG-3'</td>
</tr>
</tbody>
</table>

Fig. 1. A map of the COX-2 gene drawn to scale. Arrows, approximate locations and directions of all PCR primers within the adjacent introns. Each exon is represented by a box labeled numerically, and the size of each box is drawn to scale relative to the other COX-2 exons. Portions of the cDNA sequence that encode protein are indicated by black boxes and the untranslated portion by stippled boxes. ATG, start site for translation; TAG, stop codon for translation.

Fig. 2. Forward (5'-3') and reverse (3'-5') sequence chromatographs of the relevant region of exon 3 of COX-2 in a patient heterozygous for third bp silent mutation at nucleotide 306 (codon 102). Arrow, the position of the altered nucleotide.

Fig. 3. RT-PCR of COX-2 in patients with AAPC. PCR products were electrophoresed on a 5% 3:1 Nusieve gel and analyzed by ethidium bromide staining. To ensure that this result was quantitative, a proportional amount of sample from each tissue source was used at each step.

Three overlapping sets of primers were designed to amplify the first 1000 bases of the promoter sequence (see Fig. 4 for the approximate placement of each primer). The primer sequences were as follows: PHS2P1-F, 5'-UP-ATTTAAGCTTGAGCTG-3'; PHS2P1-R, 5'-RP-AGGTCAGAGCGGAACTCTG-3'; PHS2P2-F, 5'-UP-CTTGAAAGCTTGTGATCAGATG-3'; and PHS2P2-R, 5'-RP-ATTTAAGCTTGAGCTG-3'. PCR amplifications of the promoter region were performed as described above.
RESULTS

This study used subjects from a large pedigree with AAPC in which careful phenotypic analysis had been performed previously (23, 24). The use of subjects with an attenuated phenotype was an essential component because an accurate measurement of the number of polyps is not feasible in the typical form of APC. We began our mutational study by examining all 10 exons of the coding region of the human COX-2 gene (Fig. 1) by DNA sequence analysis in 23 patients with AAPC, 12 with low, and 11 with high numbers of colorectal polyps (25, 26). We designed intron-biased primers to amplify each exon by the PCR and identified only 1 bp change in the entire COX-2 coding sequence, a silent mutation in exon 3 at bp 306 (codon 102). Sequence chromatograms of the relevant region of exon 3 of COX-2 from a single heterozygous patient are shown in Fig. 2. In addition to the first 23 AAPC patients, we tested 33 additional AAPC subjects to see whether this polymorphism segregated with the number of colorectal polyps. Eighteen individuals with low numbers of adenomas and 15 with high numbers were homozygous for the G bp (G/G), five individuals with low numbers and four with high numbers were heterozygous (G/C) and one individual with high numbers was homozygous for the C bp (C/C); therefore, this silent mutation did not correlate with a lesser or greater number of colorectal polyps (P > 0.5, Fischer’s Exact test). Consequently, we excluded the possibility that mutations in the coding sequence of COX-2 account for a reduced tumor burden in the AAPC patients with low numbers of colorectal polyps.

We also analyzed the 3’ UTR in exon 10 of COX-2 mRNA for mutations. PCR primers were designed to amplify an AT-rich region contained within the first 200 bp of the COX-2 3’ UTR, which has been implicated in posttranscriptional control of COX-2 expression by influencing translation of the COX-2 message. We detected no bp changes in this region of the COX-2 3’ UTR.

In previous work, we showed that an increase of COX-2 mRNA in human colon cancer cells lines resulted from increased transcription of the mRNA (26). In this study, we proposed that AAPC patients with low numbers of colorectal polyps might reflect loss of transcriptional activation resulting from changes in the cis elements of the promoter region rather than mutation in the coding sequence. We tested the hypothesis that the relative levels of COX-2 mRNA may be decreased in AAPC patients who develop low numbers of colorectal polyps or increased in AAPC patients with high numbers of colorectal polyps. We designed each of the two oligonucleotide primers to cross an exon boundary so that only the cDNA, and not the genomic DNA, would be amplified. We then analyzed the level of COX-2 mRNA by RT-PCR in lymphoblastoid cells derived from one patient with a highly attenuated phenotype (six polyps at age 54), two patients with greater than 95 polyps (over the age of 50), and three normal individuals. As shown in Fig. 3, COX-2 mRNA levels were comparable among normal individuals (N1, N2, and N3), one AAPC patient with low numbers of colorectal polyps (AAPC1-Low), and two AAPC patients with high numbers of colorectal polyps (AAPC2 and AAPC3-High). Therefore, the relative level of COX-2 mRNA does not explain an increased or decreased tumor burden in AAPC patients. As controls to demonstrate specificity of the RT-PCR reaction and inducibility of the COX-2 enzyme, untreated HeLa cells [HeLa (-PE)] and HeLa cells stimulated with PE [HeLa (+PE)] were included in the mRNA analyses; the latter showed an increase in the levels of COX-2 mRNA, but untreated HeLa cells did not. Unfortunately, because the levels of COX-2 mRNA did not change in mRNA purified from normal or patient lymphoblastoid cells when treated with PE (data not shown), we were unable to examine inducibility of the COX-2 mRNA as an explanation for the differences between low and high numbers of colorectal polyps in patients with AAPC.

As an additional test of whether decreased expression of COX-2 could be involved in the attenuated phenotype, we examined the COX-2 promoter region for DNA mutations. Three sets of overlapping PCR primer sets were designed to cover the first 1000 bp of promoter sequence (Fig. 4). This portion of the COX-2 promoter contains key regulatory elements that have been defined in many promoter sequences of early-response genes and have been shown to control COX-2 expression under a variety of pathophysiological conditions (27); the figure indicates locations of the sequences that serve as binding sites for transcription factors in other genes. DNA sequence analyses in the original set of 23 AAPC patients revealed a single bp change in primer set 1 that did not correlate with the number of colorectal polyps; we tested 33 additional AAPC subjects and found the same lack of correlation with phenotype (data not shown).

DISCUSSION

COX-2, a key enzyme in prostaglandin biosynthesis, plays an important role in APC-mediated colorectal tumorigenesis in mice.

COX-2 IS NOT MUTATED IN PATIENTS WITH ATTENUATED APC

Oshima et al. (22) demonstrated that a PtgS2 null mutation dramatically reduced the number and size of intestinal polyps that developed in Apc<sup>min</sup> mice. Furthermore, treatment of Apc<sup>min</sup> mice with a COX-2 inhibitor also greatly reduced the number of intestinal polyps. Therefore, it was of considerable interest to examine the human homologue of this gene in patients carrying APC mutations responsible for AACP. We examined the COX-2 gene among two phenotypic categories of AAPC patients carrying identical mutations in the 5′ region of the APC gene, carriers with low or high numbers of colorectal polyps. Because we found no sequence alterations in either COX-2 or its promoter or any differences in expression of COX-2 mRNA that correlated with these categories, it does not appear that mutation of COX-2 is responsible for phenotypic variations among individuals affected with AAPC.

Another member of the arachidonic acid metabolism pathway, secretory phospholipase A2 (sPLA2) group Ila, Pla2g2a, has been shown to be important in determining the number of intestinal tumors that develop in Min mice (10, 28). Mutation of Pla2g2a dramatically increases the number of intestinal polyps that develop in Min mice (28), and more recently, Cormier et al. (10) have shown that Min mice carrying an overexpressing transgene containing multiple copies of Pla2g2a exhibit reduced tumor multiplicity and size relative to mice with a single copy of the gene. However, our examination of the coding sequences of the three human homologues of Pla2g2a that lie on human chromosome 1p35-36 (PLA2G2A, PLA2G2C, and PLA2G5; Ref. 29) in 20 related individuals affected with AAPC revealed no bp changes that appeared to correlate with any category of AAPC phenotype (23). This result was consistent with three other studies that failed to document the importance of sPLA2 genes at 1p35-36 in sporadic colorectal cancers (30) or inherited APC-mediated colorectal cancers (31, 32). Thus, both human and mouse studies support the conclusion that COX-2 is a necessary step in colon carcinogenesis. However, studies in humans have not yet identified genetic changes in this pathway that modify the APC phenotype.

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