Testing for Colon Neoplasia Susceptibility Variants at the Human COX2 Locus

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Background: Siblings and other first-degree relatives of patients with “sporadic” (i.e., apparently nonfamilial) colorectal cancer or precursor adenomatous colon polyps have an increased risk of developing colon neoplasia. This observation suggests the presence of inherited genetic determinants for sporadic colon neoplasia. Mice homozygous for a null cyclooxygenase 2 (COX2) (also called PTGS2) allele have a dramatically reduced susceptibility to the development of intestinal adenomas. In humans, use of pharmacologic inhibitors of COX2 enzyme activity are associated with reduced risk of colon neoplasia. This study examined whether the human COX2 locus may be linked to colon neoplasia in humans.

Methods: We used the affected sibling-pair method to test for linkage of the human COX2 locus to colon neoplasia.

Results: We examined 74 concordantly affected sibling pairs from 46 sibships with colon neoplasia. One hundred five siblings from these sibships were diagnosed with either colorectal cancer or colon adenomatous polyps before age 65 years. No linkage between COX2 and colon neoplasia was found by use of a multipoint model-free linkage analysis (estimate of allele sharing was 0.44; standard error = ±0.04; 95% confidence interval = 0.36 to 0.52). Moreover, even allowing for heterogeneity, the potential that a COX2 colon neoplasia susceptibility variant was present within a substantial subset of these sibships was strongly excluded under either a recessive or a dominant inheritance model (95% confidence to exclude a model in which 2.7% or more of the sibling pairs harbor a dominant susceptibility allele).

Conclusions: This study of concordantly affected sibling pairs thus demonstrates that variations in the COX2 gene are unlikely to be a source of individual susceptibility to colon neoplasia in humans. [J Natl Cancer Inst 2001;93:635–9]

An inherited predisposition to colorectal cancer has been associated with several highly penetrant single gene disorders that lead to colon cancer in young individuals. Relevant genes include APC, whose germline mutations account for the well-recognized familial adenomatous polyposis (FAP) syndrome, and DNA mismatch repair genes, whose germline mutations cause hereditary nonpolyposis colorectal cancer (HNPPC) (1). However, it is estimated that these syndromes account for no more than 5%–10% of all cases of colorectal cancer in humans [reviewed in (2)]. Several lines of evidence suggest that the general population harbors an unidentified susceptibility gene or genes that increase the risk of “sporadic” (i.e., apparently nonfamilial) colorectal cancer arising in typical middle-aged adults. For example, siblings and other first-degree relatives of individuals with either adenomatous polyps or colorectal cancer have a twofold to threefold elevation in risk for developing colon adenomas and cancers of the colon or rectum compared with the general population (3,4). Segregation analysis of colorectal cancer kindreds also suggests the presence of susceptibility alleles for adenomatous polyps (5,6).

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Cyclooxygenase 2 (COX2) (also called PTGS2) encodes an inducible cyclooxygenase that catalyzes the first committed step in prostaglandin synthesis [reviewed in (7,8)]. COX2 has an important role in colorectal tumorigenesis, since both the COX2 message and the protein product are increased in colon tumors and in premalignant adenomatous colon polyps (9,10). Furthermore, a reduction in COX2 activity powerfully retards the development of colon neoplasia in mice and in humans. For example, mice predisposed to develop intestinal adenomas by virtue of a heterozygous germline mutation in the APC gene show a remarkable 86% reduction in the number of tumors if they harbor allelic variants that alter the risk of colon neoplasia development.

The affected sibling-pair method is a powerful linkage technique for detecting the presence of susceptibility alleles at a given genetic locus (15,16). This method is based on the principle that two affected siblings will tend to share identical marker alleles if the marker locus is linked to the disease in question. Thus, affected siblings are statistically more likely to display increased identity by descent (IBD) at a linked locus as compared with an unlinked locus. Stated alternatively, allele sharing among concordantly affected sibling pairs should be greater than 0.5 at a marker locus linked to a disease susceptibility locus; in contrast, at any random locus, allele sharing will equal 0.5. We have employed this method to determine whether the COX2 locus itself harbors allelic variants that alter the risk of colon neoplasia susceptibility in humans.

**Patients and Methods**

**Colon Neoplasia Kindred Ascertainment**

The sibships used for this study were recruited to join the Case Western Reserve University (CWRU) Colon Neoplasia Sibling Study (CNSS), an affected sibling-pair linkage study designed to identify novel colon cancer susceptibility genes. The Institutional Review Board of University Hospitals of Cleveland approved the study and all informed consent documents. All participants reviewed and signed the informed consent document before entering the study. Selection criteria for this study included the following: 1) an index case patient and a full sibling who were both diagnosed with colorectal cancer or colon adenomatous polyps by age 65 years, 2) histologic verification of colorectal cancer or colon polyps, 3) no histologic evidence of inflammatory bowel disease, and 4) availability of a blood sample for genetic analysis. In addition, blood samples were requested from all living first-degree family members for genotyping purposes. Four-generation pedigrees were constructed for each kindred and were reviewed for known autosomal dominant forms of colon cancer, such as FAP and HNPPC (17–20). FAP kindreds were excluded by review of the clinical records for evidence of polyposis. HNPPC kindreds were excluded by microsatellite instability analysis of tumor samples from relevant cases in kindreds that met the Bethesda criteria for HNPPC (17–20).

In addition, DNA samples from all Ashkenazi Jewish patients with colorectal cancer or colon adenomatous polyps were evaluated to exclude all individuals carrying the APC 11307K allele that is found in 6% of this population and has been shown to increase the risk for colorectal cancer (17). Of the 263 probands and their kindreds reviewed for this study, 46 were eligible for genotyping and linkage analysis. Of the excluded kindreds, 206 were not eligible because of the lack of an affected full sibling of the proband or to incomplete family data, five were not eligible because of FAP, and six were not eligible because of HNPPC. No Ashkenazi sibling harbored the APC 11307K-variant allele. One hundred five affected siblings with histopathologically verified colon neoplasia and 48 additional siblings for whom no clinical colon screening information was available donated a blood sample for genotyping and statistical analysis. Twenty-five (24%) of the affected siblings were diagnosed with colorectal cancer (23 colon cancers and two rectal cancers) and 80 (76%) were diagnosed with adenomatous polyps, at an average age of 52.8 and 49.6 years, respectively. In addition, 28 (61%) of the probands were female and 54 (51%) of the siblings were female. Forty-four (96%) of the kindreds were of Northern European (Caucasian) descent.

**Genetic Analysis**

Between 15 and 20 mL of whole blood was obtained from each consenting participant, and DNA was extracted by use of standard procedures (Puregene; Gentra Systems, Inc., Minneapolis, MN). Genomic DNA (100 ng) was used in each of the 10-μL volume polymerase chain reactions (PCR) along with 2 mM deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate, 5 μM of reverse and forward primer (5′ end-labeled with T4 polynucleotide kinase and 3′-adenosine triphosphate), 50 mM KCl, 20 mM Tris–HCl (pH 8.0), 15 mM MgCl₂, and 0.75 U Taq polymerase (Life Technologies Corporation, Rockville, MD). The PCR reaction for DS1191 and DS12848 was processed on an MJ PTC-100 thermocycler (MJ Research, Inc., Waltham, MA) with a 5-minute denaturation at 96 °C, followed by a 30-second denaturation at 95 °C, a 30-second annealing at 60 °C, and a 30-second extension at 72 °C, for a total of 30 cycles. After completion, 4 μL of reaction product was added to 4 μL of formamide-containing buffer and was loaded in sequential lanes on a 6% polyacrylamide/8 M urea–sequencing gel. Reaction products along with Centre d’Etude du Polymorphisme Humain (CEPH) control DNA (Coriell Cell Repository (Camden, NJ), CEPHUTAH Pedigree 1347, R4A10859) (21) and a sequencing ladder were visualized by overnight autoradiography by use of Biomax (Eastman Kodak Co., Rochester, NY). Each autoradiograph was inspected and scored independently by two researchers before making the final allele assignment.

**Statistical Analysis**

Siblings with pathologically verified colon adenomatous polyps or colorectal cancer were scored as affected. Other siblings without colon screening were scored as unknown affected state. The genotypes from parents and the other siblings were used to better define the parental haplotypes of markers flanking the COX2 locus and to improve the determination of parental allele transmission. All possible distinct-affected sibling pairs were used from the triplet and quadruplet sibships. Marker alleles for each affected sibling can be shared or not shared, π, or the proportion of alleles shared IBD by an affected-sibling pair, was estimated by use of the GENIBD and analyzed by use of the SIBPAL2 multipoint linkage programs in the program package Statistical Analysis for Genetic Epidemiology (S.A.G.E.) that is based on the methods of Idury, Elston, and Haseman (22–24). π would equal 0.5 under no linkage and would be greater than 0.5 if COX2 were linked to colon neoplasia. Under the null hypothesis of no linkage, the allele sharing from different pairs in a sibship is pairwise independent and thus can be treated as independent events for the mean test (16,25). Map distances between the two flanking polymorphic dinucleotide repeat markers DS1191 and DS12848 were estimated to be 0.6 and 1.4 cM (26). The polymorphism information contents for markers DS1191 and DS12848 were 0.74 and 0.84, respectively.

The transmission of a preferential parental allele was analyzed by use of the global exact transmission–disequilibrium (TDTEx) program in S.A.G.E. and by the method of Spielman and Ewens (27,28) and McGinnis et al. (29). The TDTEx program analyzes all marker alleles simultaneously to discern preferential transmission of any allele to the affected siblings, so there is no need for additional correction because of multiple tests. The transmission–disequilibrium test (TDT) and the sibling–TDT (S-TDT) are methods that examine each allele separately, allowing for the development of a statistic that tests whether specific alleles are associated with the disease (30). Confidence for excluding a COX2 susceptibility allele present in a fraction (λ) of the
population was based on the normal distribution of the estimate of IBD sharing ($\hat{\tau}$) and a one-sided test. For example, in a reasonably large sample, $\pi$ can be estimated as $\hat{\tau}$, with a standard error of $s$, so that $P(\pi < \hat{\tau} + 1.65s) = 0.95$ for a one-sided interval.

**RESULTS**

The degree of allele sharing at the COX2 locus was studied in siblings from 46 sibships that were identified from the CWRU CNSS. Each of the 46 sibships included at least two concordantly affected siblings in whom colon neoplasia had been diagnosed at or before the age of 65 years. In total, the affected sibships included 105 affected individuals and defined 74 concordantly affected sibling pairs. Eighty individuals (76%) were affected with adenomatous colonic polyps and 25 (24%) were affected with colorectal cancer. Thirty-five sibships had two affected siblings, nine had three, and two had four. Fig. 1 summarizes the structure of the affected sibships studied. We specifically excluded from this analysis any sibling pairs from kindreds demonstrating HNPCC or FAP and any sibship demonstrating the presence of the APC II1307K variant observed among some Ashkenazi Jews (17).

**COX2 Locus and Colon Neoplasia Susceptibility**

Genotypes were determined for two highly polymorphic microsatellite markers, D1S191 and D1S2848, that flank the COX2 locus at a distance of 0.6–1.4 cM (26). With the use of the haplotypes formed by these two loci, more than 90% of the individuals studied were fully informative for two distinguishable COX2 alleles. Genotypes of the COX2 locus markers were determined for each of the 105 affected siblings within the 46 sibships. In addition, DNA samples from 48 siblings of unknown affection status and 21 parents were genotyped. The degree of allele sharing because of IBD among the concordantly affected sibling pairs was determined by use of a model-free multipoint linkage analysis carried out in the GENIBD program within S.A.G.E. (24). For these 74 sibling pairs, the multipoint estimates of IBD allele sharing ($\hat{\tau}$) were 0.44 for both the centromeric marker D1S191 and the telomeric marker D1S2848, with a calculated standard error of 0.04 for both estimates (Table 1). In a model-free analysis, linkage to a candidate susceptibility locus is recognized by an IBD estimate greater than 0.5. Our finding a $\hat{\tau}$ less than 0.5 (0.44; standard error = ±0.04; 95% confidence interval = 0.36 to 0.52) excludes with 93% confidence that any future study of the COX2 locus would yield an IBD greater than 0.5 (Table 1). This estimate effectively excludes the possibility of COX2 colon neoplasia susceptibility variants segregating in the human population.

A more intuitive understanding of the strength of our results can be achieved by assuming that a recessively acting COX2 colon neoplasia susceptibility variant does account for the colon neoplasia in each of our affected sibships. In this case, the expected allele sharing ($\hat{\tau}$) would be 1.0. Our measured $\pi$ of 0.44 completely excludes this model. Similarly, if we assumed that a dominant COX2 neoplasia susceptibility variant accounts for all colon neoplasia in each of our affected sibships, the expected allele sharing in the affected sibling pairs would be 0.75. In this case, our measured $\pi$ of 0.44 ± 0.04 excludes this model with a one-sided $P$ value of less than $5 \times 10^{-10}$.

The frequencies of the different alleles of markers D1S191 and D1S2848 that we observed in these sibships were similar to both published values and to frequencies that we observed in a set of 100 control chromosomes (data not shown). However, our conclusion that the COX2 locus is not linked to colon neoplasia susceptibility is not dependent on our use of published population frequencies for the different microsatellite alleles. Indeed, $\pi$ determined for the COX2 locus among our sibling pairs remained less than 0.5 when the analysis was repeated, assuming that all of the alleles at D1S191 and D1S2848 were equally frequent, or, alternatively, when the relative frequency of each allele was made proportional to its complement, i.e., $1 - p_i$, where $p_i$ is the published frequency.

**Evaluation of COX2 Susceptibility Alleles in Subpopulations of Sibships With Colon Neoplasia**

Our measured $\pi$ of 0.44 ± 0.04 also effectively excludes models in which colon neoplasia susceptibility would be linked to the COX2 locus among any substantial subpopulation of sibling pairs from which our sibships were sampled. For example, assume that these sibling pairs are composed of two subgroups, one accounting for a proportion $(1 - \lambda)$, in which COX2 susceptibility variants are not present (i.e., in which $\pi$ for COX2 = 0.5), and a second accounting for a proportion of $\lambda$, in which a dominant or recessive COX2 susceptibility variant is present. For a dominantly acting allele in the subset of size $\lambda$, the $\pi$ IBD sharing of the total sibling-pair population would be $0.5(1 - \lambda) + 0.75\lambda$, or $0.5 + 0.25\lambda$, reflecting a $\pi$ of 0.5 in the unlinked subset and 0.75 in the subset with linkage. If a recessive COX2 susceptibility variant were present in a subset of size $\lambda$, the IBD for the total sibling-pair population would be $0.5 + 0.5\lambda$, reflecting a $\pi$ of 1.0 and 0.5 for the linked and unlinked subsets, respectively. As shown in Table 2, the observed data exclude with 95% confidence a model in which 2.7% or greater of sibling pairs harbor a dominant COX2 susceptibility allele. Our data similarly exclude with 99% confidence any model.

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Fig. 1. Shown is the family structure of each of 46 full sibships genotyped with the cyclo-oxygenase 2 (COX2)-flanking markers, D1S191 and D1S2848. The majority (n = 35) of the sibships consisted of two concordantly affected full siblings with colorectal cancer or adenomatous polyps (n = 19) or both siblings with adenomatous polyps (n = 16). Nine sibships had three concordantly affected siblings, and two had four concordantly affected siblings. Solid diamond = male or female with colon cancer; shaded diamond = male or female with adenomatous polyps; number in parentheses = number of sibships in each category.

<table>
<thead>
<tr>
<th>Marker</th>
<th>$\pi$</th>
<th>SE</th>
<th>95% CI</th>
<th>$P$</th>
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<tbody>
<tr>
<td>D1S191</td>
<td>.406</td>
<td>.04</td>
<td>.08 .93</td>
<td></td>
</tr>
<tr>
<td>D1S2848</td>
<td>.431</td>
<td>.04</td>
<td>.08 .92</td>
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</table>

*Model-free multipoint linkage analysis of 74 affected sibling pairs with colon adenomatous polyps or colorectal cancer. One-sided $P$ values were nonsignificant for linkage for each flanking the cyclo-oxygenase 2 marker. $P$ values were calculated from the estimate of allele sharing due to identity by descent ($\hat{\tau}$) and its associated standard error (SE) and 95% confidence interval (CI).
in which 13% or more of our sibling-pair population harbors a dominant susceptibility allele. For a recessive susceptibility allele, we similarly exclude with 99% confidence any model in which 7% or more harbor a susceptibility allele. Thus, we can exclude with high confidence the hypothesis that a COX2 susceptibility allele is a determinant of colon neoplasia susceptibility in any substantial subgroup of the population studied.

This conclusion is supported independently by our analysis of these sibling pairs by use of the TDT and the S-TDT. These tests search for linkage of colon neoplasia susceptibility to the COX2 locus by examining the number of transmis-

**Table 2.** Confidence levels for excluding the presence of a cyclooxygenase 2 susceptibility variant in a subset greater than or equal to a proportion λ of the sibling pairs concordantly affected by colon neoplasia

<table>
<thead>
<tr>
<th>Autosomal recessive</th>
<th>Autosomal dominant</th>
</tr>
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<tbody>
<tr>
<td>λ</td>
<td>Confidence excluded at</td>
</tr>
<tr>
<td>0.0133</td>
<td>0.5054</td>
</tr>
<tr>
<td>0.0670</td>
<td>0.5323</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Marker</th>
<th>No. of siblings informative for marker</th>
<th>(P^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1S191</td>
<td>38</td>
<td>.93</td>
</tr>
<tr>
<td>D1S2848</td>
<td>41</td>
<td>.88</td>
</tr>
</tbody>
</table>

*P values reflect simultaneous testing of all alleles (seven alleles for D1S191 and 11 for D1S2848).

**DISCUSSION**

We find that the COX2 locus is highly unlikely to harbor colon neoplasia susceptibility variants in any substantial fraction of the general human population. This conclusion is based first on a model-free analysis demonstrating an absence of linkage of colon neoplasia susceptibility to the COX2 locus in 74 sibling pairs concordantly affected with colon cancer or adenomatous polyps. Moreover, we have developed a simple test statistic, λ, calculated from our estimate of IBD sharing (\(\hat{\theta}\)) and from the standard error of that estimate, that allows us to exclude—with a high degree of confidence—linkage of colon neoplasia to COX2 in any substantial subpopulation of the sibships participating in our study. The results of our study are supported independently by the lack of detecting disease susceptibility alleles by use of a TDT, which is a powerful test of linkage to a candidate gene. Thus, while activity of the COX2 gene product is clearly required for the development of colon neoplasms, inherited COX2 alleles that increase the risk for intestinal neoplasia are unlikely to be a feature of the human population. We note that a common COX2 resistance variant that decreased colon neoplasia risk would also likely be detected in a study of affected sibling pairs, but that greater power for observing such a resistance variant would be obtained in an alternate design that employed concordant-unaffected sibling pairs. However, studies of attenuated phenotypes in some familial polyposis kindreds previously have not found evidence for such COX2 resistance variants acting in humans (33).

In addition to excluding COX2 as an important human colon neoplasia susceptibility locus, this study illustrates the utility of the affected sibling-pair method for approaching the general problem of evaluating putative colon neoplasia susceptibility genes. One advantage of the affected sibling-pair linkage study design is that the flanking markers used to mark a given locus act as surrogates to test for disease association with all potential polymorphisms contained within the given genetic locus, including the coding region, promoters, and noncoding regions of the gene. Additionally, the affected sibling-pair study design yields conclusions more applicable to the general population of individuals with colon neoplasia and not just to potentially atypical highly selected kindreds. Moreover, experimental study designs that compare affected case patients versus control populations may be influenced by selection differences between case patients and control subjects with regard to ethnic background or other parameters. The affected sibling-pair method used here avoids this source of error by use of a disease-aﬀected population only. Last, the affected sibling-pair method allows testing genetic linkage to diseases, such as sporadic colon neoplasia, for which disease onset is later in life and for which multigenerational kindreds may be rare. The population of affected sibling pairs employed in this study will thus be useful for the evaluation of other candidate loci as well as for supporting a future whole genome scan for detecting novel colon neoplasia susceptibility loci.

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