Familial adenomatous polyposis (FAP) is an autosomal dominant condition caused by inherited mutations in the adenomatous polyposis coli (APC) or in the MYH genes. Clinical trials have established that nonsteroidal anti-inflammatory drugs (NSAID) are effective in preventing the development as well as reducing the size and decreasing the number of adenomas in FAP patients. Our aim was to evaluate the cyclooxygenase-2 (COX-2) expression in surgical specimens from patients with no evidence of germ line APC mutations but carrying germ line MYH mutations. COX-2 expression was evaluated through immunohistochemical and mRNA analysis in carcinomas, adenomas, and healthy mucosa from six patients carrying germ line biallelic MYH mutations.

Introduction

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited condition that predisposes to the development of hundreds to thousands of colorectal adenomas, some of which progress to cancer. This syndrome is caused by inherited mutations in the adenomatous polyposis coli (APC) gene, which lead to a deregulated proliferation of colonic cells (1). Attenuated FAP (AFAP), characterized by smaller numbers of polyps (<100) or by diagnosis at older age, is typically associated with germ line mutations in the extreme 5' or 3' ends of the APC gene (1). However, not all phenotypically FAP and AFAP patients show detectable germ line APC mutation.

Recently, germ line biallelic MYH mutations have been reported in "APC-negative" FAP and AFAP patients with no evidence of vertical transmission of the disease (2–4). MYH is a base excision repair gene which removes adenines mispaired with 8-oxo-7,8-deoxyguanosine (a particularly stable product of oxidative DNA damage) preventing somatic G:C → T:A transversion mutations (5). Tumor cells of MYH-associated polyposis (MAP) therefore contain an excess of somatic G:C → T:A transversions, mainly in the APC gene (2, 6). It is possible that MAP-associated tumorigenesis preferentially involves a subset of genes/mutations in target genes that confer different biological properties to MAP adenoma and/or carcinoma.

A modulation of COX-2 expression from adenoma (lower level) to carcinoma (higher level) was observed in all patients by both immunohistochemical and mRNA analysis. Moreover, patients with MYH mutations showed a weak COX-2 expression in the whole colorectal mucosa, as for classic FAP patients carrying germ line APC mutations. All together, our data suggest that biallelic MYH patients might benefit from NSAID treatment, because in these patients COX-2 is overexpressed in the whole colorectal mucosa, a finding possibly related to the interplay between COX-2 and APC protein being the APC gene a common target of mutations in MYH patients.

Materials and Methods

Patients. We analyzed the germ line APC mutational status in a series of Italian patients (n = 280) reported in the
Hereditary Colorectal Tumor Registry of the National Cancer Institute of Milan with a diagnosis of FAP or AFAP and verified from both medical records and family history. The diagnosis of FAP or AFAP was made in the presence of multiple synchronous or metachronous (≥100 or <100, respectively) colorectal adenomas. We selected patients (n = 80) where a germ line APC mutation was not detected (data not shown). In this subgroup of patients, we identified and investigated six FAP patients (Table 1), carrying MYH germ line mutations, belonging to five different families (patients 3 and 4 belong to the same family), of which formalin-fixed, paraffin-embedded and, whenever possible, frozen material were available. The MYH germ line profile of five (patients 1, 2, 3, 5, and 6) of these patients was already reported (4). The main clinicopathologic data are reported in Table 1. All patients had given written informed consent for the testing of blood DNA samples according to protocols approved by the ethical committee. For comparison, 35 colorectal cancer from FAP patients carrying germ line APC mutation and 40 patients with sporadic colorectal cancer (SCRC) were also analyzed.

DNA and RNA Extraction. Constitutional DNA was extracted from peripheral blood lymphocytes using a standard proteinase K and phenol-chloroform protocol as already reported (13).

Total RNA was extracted with the RNAzol method (Life Technologies, Grand Island, NY) after microdissection of tumoral and normal frozen tissues assessed by H&E section. Only cancers containing at least 70% of neoplastic cells were processed for the study. Normal mucosa from FAP and MAP patients were carefully analyzed by microscopic evaluation and the presence of microadenomas was excluded. One microgram of total RNA was used for reverse transcription-PCR according to the manufacturer’s instructions. A housekeeping gene, β-actin, was used as internal control to check the quality of RNA extracted and the efficiency of cDNA synthesis and PCR amplification. One microliter of cDNA was used as template for each PCR reaction.

APC Gene Analysis. Germ line–truncating mutations were investigated by single-strand conformation polymorphism analysis for DNA sequences corresponding to APC exon 1 to 14 and exon 15 fragments A, E, and G according to the literature (13). APC exon 15 was completely analyzed through the protein truncation test as already reported (13). DNA fragments with an abnormal single-strand conformation polymorphism or protein truncation test pattern were subjected to automated sequencing by ABI Prism 377 (Applied Biosystems, Foster City, CA) and analyzed with Sequencing Analysis and Sequence Navigator software programs by ABI Prism (Applied Biosystems).

MYH Gene Analysis. Germ line MYH hotspots in exons 7, 13, and 14 and the whole coding region of the MYH gene were analyzed as already reported (4). Exon 7, in which Y165C mutation is located, was examined in all samples by direct sequence and amplification refractory mutation system, described by Al Tassan et al. (2). Exon 13, in which G382D mutation is located, was examined in all samples by direct sequence or BglII digestion. Exon 14, in which 1395delGGA is located, was examined on an ABI PRISM 310 Genetic Analyzer using GeneScan Analysis. The presence of 1395delGGA was confirmed by sequence analysis.

COX-2 Immunohistochemistry. COX-2 immunophenotyping was carried out in formalin-fixed, paraffin-embedded samples. All specimens were handled identically in respect of postoperative management (time before fixation, type and time of fixation). Sections were cut and treated according to the protocol already reported (14). As a primary antibody, a mouse monoclonal anti-COX-2 antibody (Cayman Chemical, Ann Arbor, MI) was diluted at 1:50 in a solution containing 0.05 mol/L PBS, 1% bovine serum albumin, and 0.1% sodium azide. As negative control, we preincubated the setting-up working dilution of primary COX-2 antibody in an appropriate blocking buffer with the blocking peptide (Cayman Chemical) according to manufacturer’s instructions and then we did the immunohistochemical staining according to the protocol abovementioned. The blocking peptide is derived from the human COX-2 sequence (15). A colorectal cancer sample previously displaying COX-2 overexpression at cytoplasmic and, occasionally, at nuclear membrane level was considered as positive control.

COX-2 mRNA Expression. The analysis of mRNA expression was done on the basis of the protocol already reported in the literature (16). Amplified cDNA products of 724 bp were run on 2% agarose gel, stained with ethidium bromide, and visualized under UV light.

Results

COX-2 Immunophenotyping. COX-2 expression was observed mainly in the cytoplasm and, occasionally, at outer nuclear membrane. A strongly enhanced COX-2 immunoreactivity was detected in carcinomas, in comparison with adenomas and surrounding healthy mucosa from the same patient. Furthermore, in all MAP patients the COX-2 protein was more expressed in adenomas than in normal mucosa specimens (Fig. 1). More in detail, all healthy mucosa specimens from MAP patients, both homozygous and heterozygous compounds, displayed a weak COX-2 immunoreactivity close to the adenoma (Fig. 1A-B) and at the resection margin. The same COX-2 immunohistochemical pattern detected in MAP patients was found in a series of classic FAP patients carrying a germ line APC mutation (35 samples; data not shown). An example is reported in Fig. 1C. By contrast, in SCRC patients (40 samples), tumor specimens showed a strong

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Total number of adenomas</th>
<th>CRC at diagnosis or follow-up</th>
<th>Surgical treatment</th>
<th>APC germ line mutation</th>
<th>MYH germ line mutation</th>
<th>COX-2 normal mucosa (IHC)</th>
<th>COX-2 adenomas/carcinoma (IHC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>57</td>
<td>90</td>
<td>Yes</td>
<td>IRA</td>
<td>wt</td>
<td>Y165C/Y165C</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>46</td>
<td>&gt;100</td>
<td>Yes</td>
<td>ISA</td>
<td>wt</td>
<td>1395 del GGA/</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>3d</td>
<td>F</td>
<td>37</td>
<td>&gt;100</td>
<td>Yes</td>
<td>IRA</td>
<td>wt</td>
<td>1395 del GGA/</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>51</td>
<td>&gt;100</td>
<td>Yes</td>
<td>ISA</td>
<td>wt</td>
<td>1395 del GGA/</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>58</td>
<td>&gt;100</td>
<td>No</td>
<td>IRA</td>
<td>wt</td>
<td>G382D/1395 del GGA</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>49</td>
<td>Numerous</td>
<td>Yes</td>
<td>IRA</td>
<td>wt</td>
<td>G382D/1395 del GGA</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

Abbreviations: IRA, ileo-rectal anastomosis; ISA, ileo-sigmoid anastomosis; wt, wild type; IHC, immunohistochemistry; CRC, colorectal cancer.

*Patients belonging to the same family.
COX-2 expression, whereas the surrounding healthy mucosa was faintly or null COX-2 expressing and resection margins were completely immunonegative (an example is depicted in Fig. 1D).

**COX-2 mRNA Expression.** The differences in COX-2 expression between tumor and healthy mucosa from MAP, FAP, and SCRC patients were confirmed by RNA expression analysis. Unfortunately, frozen material for RNA evaluation was available in one only MAP patient (patient 3, carrying a homozygous germ line MYH mutation). As depicted in Fig. 2A, tumor specimens from MAP, FAP, and SCRC patients display a strong COX-2 mRNA expression in comparison to healthy mucosa. It is noteworthy that normal mucosa from the MAP patient expresses a low level of COX-2 mRNA, similarly to that observed in the two FAP patients reported (Fig. 2A) and differently from SCRC patients, whose normal mucosa was completely negative. The differences in COX-2 mRNA among the samples depend on a modulation typical of COX-2, as the expression of β-actin gene is similar in all samples (Fig. 2B).

**Discussion**

Randomized clinical trials and observational studies have proved that NSAIDs, molecules recognized to be able to inhibit the COX enzyme family, may help the management of patients with FAP (8-10). Recently, a FAP-like condition was found to occur in patients with germ line biallelic MYH mutations, in addition to patients carrying germ line APC mutations (2). We analyzed the COX 2 expression, both at

![Figure 1. Immunohistochemical staining of COX-2 protein in patients carrying germ line MYH mutations [homozygous (A), heterozygous compounds (B)], APC mutation (C), or with SCRC (D). A, adenoma (top) and normal mucosa (bottom). B-D, adenoma (left) and normal mucosa (right).](image)

![Figure 2. COX-2 mRNA expression in FAP patients and in a biallelic MAP and SCRC patient. N, normal mucosa; T, tumor lesion. Bottom, the same RNA samples were analyzed for the expression of a housekeeping gene, β-actin, as internal control to check the quality of RNA extracted and the efficiency of cDNA synthesis and PCR amplification.](image)
immunohistochemical and mRNA levels, in MAP patients, both homozygous and heterozygous compounds, to evaluate its expression pattern in MAP tumors as well as in healthy mucosa. Our results indicate that an increase of COX-2 expression can be observed in MAP tumors, both adenoma (lower level of expression) and carcinoma (higher level of expression). Moreover, we found that in all specimens COX-2 expression was also present in areas surrounding the malignant lesion as well as at the resection margin.

The pattern of COX-2 expression in healthy mucosa from MAP patients mirrors that observed in classic FAP patients carrying a germ line APC mutation (data not shown and already reported; ref. 12). The finding is perhaps not surprising because this pattern may be explained by the interplay between COX-2 and APC protein and because the APC gene seems a common target of somatic mutations in MAP tumorigenesis. Preclinically, wild-type APC protein down-regulates COX-2 protein expression and leads to β-catenin degradation, whereas when a germ line and/or somatic mutation occurs, COX-2 and β-catenin are overexpressed (17, 18). Accordingly, in clinical setting, after treatment with sulindac (a COX-2 inhibitor) a strong decreasing of nuclear β-catenin overexpression was observed (19). As MYH mutated patients show a relevant number of somatic G:C → T:A transversions in the APC gene (2, 6), it is conceivable that in cases carrying biallelic germ line MYH mutations, similarly to classic FAP patients, a high mutational pressure on the APC gene could occur, leading to COX-2 expression in tumoral and nontumoral colorectal mucosa. This is at variance with sporadic colorectal cancer where COX-2 expression is limited to neoplastic lesions and normal mucosa is APC and MYH negative as well as null for COX-2 expression.

In conclusion, the present data, which have to be confirmed by the analysis of larger case material, suggest that MYH patients, both homozygous and heterozygous compounds, might benefit of NSAID treatment.

Acknowledgments
We thank Mariangela Di Ceglie and Anna Maria Ghinatti for secretarial assistance.

References

Cancer Epidemiol Biomarkers Prev 2005;14(8). August 2005
Downloaded from cebp.aacrjournals.org on June 15, 2017. © 2005 American Association for Cancer Research.
Cancer Epidemiology, Biomarkers & Prevention

Cyclooxygenase-2 Expression in FAP Patients Carrying Germ Line MYH Mutations

Milo Frattini, Ileana Carnevali, Stefano Signoroni, et al.

Cancer Epidemiol Biomarkers Prev 2005;14:2049-2052.

Updated version
Access the most recent version of this article at:
http://cebp.aacrjournals.org/content/14/8/2049

Cited articles
This article cites 19 articles, 7 of which you can access for free at:
http://cebp.aacrjournals.org/content/14/8/2049.full#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cebp.aacrjournals.org/content/14/8/2049.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.