LETTER TO JMG

Genetic characterisation of patients with multiple colonic polyps

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The aim of the present study was to evaluate in a group of patients with a marked excess of colorectal adenomas, often associated with carcinomas, whether a genetic defect could be detected in one of the two major pathways responsible for colorectal tumorigenesis, namely the WNT signal transduction pathway and DNA mismatch repair. We have analysed microsatellite instability as well as immunoeexpression of MMR proteins (MSH2, MLH1, and MSH6) and of β-catenin. Germline mutations in APC or in the MMR genes have been investigated in those cases where the MSI and/or IHC analyses were suggestive of a defect in either WNT signal transduction or mismatch repair pathways.

MATERIAL AND METHODS
Patient selection
The study was approved by the local ethics and scientific committees and informed written consent was obtained from living patients entering the study.

Sixteen patients with a number of adenomas varying between six and 50 were included in this study; 8/16 patients also presented with synchronous or metachronic colorectal carcinomas, whether a genetic defect could be associated with carcinomas, whether a genetic defect could be detected in one of the two major pathways responsible for colorectal tumorigenesis, namely the WNT signal transduction pathway and DNA mismatch repair. We have analysed microsatellite instability as well as immunoeexpression of MMR proteins (MSH2, MLH1, and MSH6) and of β-catenin. Germline mutations in APC or in the MMR genes have been investigated in those cases where the MSI and/or IHC analyses were suggestive of a defect in either WNT signal transduction or mismatch repair pathways.

Microsatellite instability/LOH analysis
For each sample (adenoma or carcinoma), 10 sections of 10 µm each were cut parallel to the section used for histological classification and the DNA was extracted from paraffin embedded tissues using a proteinase-K digestion method followed by phenol-chloroform extraction. Constitutional DNA was isolated from peripheral blood using a salting out procedure.

DNA was PCR amplified at different loci (D2S123, D5S346, BAT-25, BAT-26, and TP53). Briefly, DNA was amplified by PCR with the incorporation of [32P]-dCTP. PCR products were diluted with a denaturing buffer (95% formamide, 0.01% xylene cyanol, 0.01% bromophenol blue, 20 mmol/l NaOH) and subsequently denatured and electrophoresed on polyacrylamide gels containing 6.9 mol/l urea and 32.5% formamide. Gels were exposed overnight to MP film (Amersham Corp, UK) at −70°C.

Microsatellite instability was defined as the presence of novel alleles observed in neoplastic DNA but not present in the corresponding normal DNA.

Abbreviations:
FAP, familial adenomatous polyposis coli; HNPCC, hereditary non-polyposis colorectal cancer; AAPC, attenuated FAP; MMR, mismatch repair; MSI, microsatellite instability; CRC, colorectal cancer; PTT, protein truncation test; DGGE, denaturing gradient gel electrophoresis.
Table 1 Clinical characterisation of patients included in the study and clinical and pathological characterisation of carcinomas and adenomas

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at diagnosis (y)</th>
<th>Familial history (CRC)</th>
<th>No of carcinomas</th>
<th>No of adenomas</th>
<th>Localisation of adenomas</th>
<th>Size of adenomas (cm)</th>
<th>Histological characterisation of the adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>75</td>
<td>No</td>
<td>1 (right colon)</td>
<td>20–50</td>
<td>Whole colon &lt;2</td>
<td>TA (LGD and HGD)</td>
<td>TA (LGD and HGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>No</td>
<td>1</td>
<td>29</td>
<td>Whole colon 0.2–2</td>
<td>TA (LGD)</td>
<td>TA (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>3</td>
<td>66</td>
<td>No</td>
<td>2 (right colon)</td>
<td>6</td>
<td>Whole colon –</td>
<td>TA and TV (LGD and HGD)</td>
<td>TA and TV (LGD and HGD)</td>
</tr>
<tr>
<td>4</td>
<td>70</td>
<td>No</td>
<td>0</td>
<td>12</td>
<td>Whole colon –</td>
<td>TA and TV (LGD and HGD)</td>
<td>TA and TV (LGD and HGD)</td>
</tr>
<tr>
<td>5</td>
<td>44</td>
<td>No</td>
<td>1 (right colon)</td>
<td>50</td>
<td>Whole colon 0.5–1</td>
<td>TA (LGD)</td>
<td>TA (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>6</td>
<td>35</td>
<td>No</td>
<td>0</td>
<td>20–30</td>
<td>Whole colon 0.5–3</td>
<td>TA and TV (LGD)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
<td>No</td>
<td>0</td>
<td>30</td>
<td>Whole colon 1–4</td>
<td>TA and TV (LGD and HGD)</td>
<td>TA and TV (LGD and HGD)</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>No</td>
<td>0</td>
<td>40–50</td>
<td>Whole colon 0.2–2</td>
<td>TA (LGD and HGD)</td>
<td>TA and TV (LGD and HGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>9</td>
<td>57</td>
<td>No</td>
<td>0</td>
<td>28</td>
<td>Whole colon 0.5–2</td>
<td>TA (LGD and HGD)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>10</td>
<td>48</td>
<td>Yes</td>
<td>1 (rectum)</td>
<td>30–40</td>
<td>Whole colon &lt;1</td>
<td>TA (LGD)</td>
<td>TA (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>11</td>
<td>69</td>
<td>No</td>
<td>1 (right colon)</td>
<td>43</td>
<td>Whole colon 0.5–2</td>
<td>TA and TV (LGD)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>No</td>
<td>1 (right colon)</td>
<td>30</td>
<td>Whole colon –</td>
<td>TA and TV (LGD)</td>
<td>TA and TV (LGD and HGD)</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>No</td>
<td>1 (left colon)</td>
<td>8</td>
<td>Whole colon 0.2–1.4</td>
<td>TA and TV (LGD)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>14</td>
<td>73</td>
<td>Yes</td>
<td>0</td>
<td>13</td>
<td>Right colon 0.3–2</td>
<td>TA (LGD), hyperplastic (n=2) and serrated polyps (n=1)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
<tr>
<td>15</td>
<td>34</td>
<td>Yes</td>
<td>0</td>
<td>7</td>
<td>Whole colon 0.4–2</td>
<td>TA and TV (LGD and HGD) and hyperplastic polyps (n=1)</td>
<td>TA and TV (LGD) and hyperplastic polyps (n=1)</td>
</tr>
<tr>
<td>16</td>
<td>70</td>
<td>Yes</td>
<td>1 (left colon, mucinous)</td>
<td>9</td>
<td>Left colon 0.2–3</td>
<td>TA and TV (LGD) and hyperplastic polyps (n=1)</td>
<td>TA and TV (LGD) and hyperplastic polyps</td>
</tr>
</tbody>
</table>

TA, tubular adenoma; TVA, tubulovillous adenoma; LGD, low grade dysplasia; HGD, high grade dysplasia.

Mutational analysis

For APC mutation analysis of exon 15, the protein truncation test (PTT) was used as described by Powell et al. Exon 15 was divided into four overlapping fragments that were PCR amplified. The 5′ primers used for amplification of these fragments have in their 5′ end a T7 promoter sequence used for transcription initiation as well as a sequence for translation onset. In vitro translated proteins were then separated on a 14% gradient SDS-polyacrylamide gel and visualised after autoradiography. Exons 1-14 of the APC gene were analysed using denaturing gradient gel electrophoresis (DGGE) with one of the primers containing at its 5' end a GC rich sequence (GC clamp) to increase screening efficiency. The amplified product was then loaded on a 6% polyacrylamide gel containing a linearly increasing denaturant gradient (100% denaturant=7 mol/l and 40% for-mamid (v/v); acrylamide:bisacrylamide=39:1). The electrophoresis was performed at 160 V in TAE buffer (40 mmol/l Tris-acetate pH 7.5, 20 mmol/l sodium acetate, 1 mmol/l Na, EDTA) at a constant temperature of 60°C for four hours. Finally, the gel was stained with ethidium bromide and visualised under UV light. DGGE was also used to analyse fragment G from exon 15 (codons 1263-1377) using previously described primers.

Mutation analysis in MMR genes was performed using DGGE with previously described primers. Whenever there was a pattern corresponding to the normal pattern of expression, β-catenin expression was evaluated by the transition from membrane expression, occurring in normal cells, to nuclear expression. The nuclear expression was scored as −, +, ++, and ++++, according to the percentage of cells expressing β-catenin in the nucleus; absence of nuclear expression (−), less than 25% (+), 25-50% (++), and more than 50% (+++).

RESULTS

The clinicopathological and family history data of the patients included in the present study are summarised in table 1. The cohort included 10 males and 6 females and the mean age at diagnosis was 60. The patients’ age at diagnosis ranged from 34 to 75 years. The median number of colonic adenomas was 25 (range six to 50). None of the patients had features of classical FAP. However, there were also eight patients who presented with 11 colorectal carcinomas, most of them located in the right colon (8/11 tumours), a few of them arising in the fourth decade of life, and others associated with a lower number of colonic adenomas. Thus, although the family history was in all but four patients, the study group presented with clinical features that could be suggestive of attenuated or atypical FAP or HNPCC.

Histological characterisation of colonic polyps showed that most lesions analysed were tubular or tubulovillous adenomas, except for patient 11 where the large majority of polyps were hyperplastic lesions (table 1).

Table 2 summarises the results obtained for the colonic tumours analysed. In most cases two or more specimens were analysed per patient. When a carcinoma was present it was always included in the analysis. With respect to MSI analysis, all the carcinomas and adenomas examined were scored as MSS. Use of these markers also showed that 7/16 (44%) patients showed LOH or allelic imbalance at several loci (D2S123, D5S346, and TP53) (table 2). This was observed in 4/8 carcinomas and in 8/25 adenomas analysed. Fig 1 shows LOH for D2S123 and D5S346 in the carcinoma from patient 2. To confirm that most of these lesions did not arise from MMR mutations, expression of MSH2, MLH1, and MSH6 was evaluated by immunohistochemical analysis of all the available tumours. Normal expression patterns were observed in all
cases with the exception of the carcinomas from patients 5 and 13 where no MSH2 and MSH6 expression was detected. The adenomas analysed for these patients showed a normal expression of these proteins, thus there is no indication that the adenomas show any defect in the MMR genes. In order to evaluate the presence of any possible germline defect, germline mutations in the corresponding MMR genes were analysed in these patients. A missense mutation (GGC → GAC) was found in codon 322 of MSH2 exon 6 in patient 5. This results in substitution of the amino acid glycine for aspartate. Germline variants at this codon have been detected in HNPCC patients but also in apparently normal subjects.21 22

MSI was not observed at any of the markers analysed in the corresponding carcinoma, though MSI-H is only observed in 95% of HNPCC associated tumours. The absence of MSI was further evaluated by using the markers BAT-40 and MYCL that are more specific for analysing the presence of MSI-L.

Since the majority of the lesions analysed in this series seemed to follow the suppressor pathway (frequent LOH, absence of MSI, and normal expression of MMR proteins in the great majority of tumours), we set out to search for germline mutations in the entire coding region of the APC gene in the 16 patients. No mutations were detected in any of the patients included in the study. Because PTT does not detect I1307K and E1317Q variants, codons 1263-1377 were analysed by DGGE in each of the patients but again no mutations were found (data not shown).

As shown in table 2, nuclear expression of β-catenin was detected in the vast majority of tumours analysed: in 20/26 adenomas and 7/8 carcinomas. Fig 2B shows an example of membrane and nuclear β-catenin expression in one carcinoma. Although the numbers are clearly too small, we observed a tendency for higher β-catenin expression in adenomas with a high grade dysplasia as compared to...
DISCUSSION

There is increasing evidence that some patients with colorectal tumours have features of both FAP and HNPCC. A few published studies have searched for potential germline defects in CRC predisposing genes. Results published so far are conflicting, because while some studies reported germline variants of the APC gene that could predispose to the development of multiple colorectal adenomas and carcinoma,\(^\text{11,12}\) others could not find mutations in this gene.\(^\text{13}\) Moreover, germline HNPPCC mutations of uncertain functional effect were found in a minority of the patients analysed by Beck et al.\(^\text{14}\) Most patients included in the present series had a clear excess of colorectal adenomas, which could suggest an APC related phenotype. However, a substantial number of them also presented with colorectal cancers predominantly located in the right colon, suggestive of a HNPPCC-like syndrome.

Notably, the majority of these patients do not have a family history of colorectal cancer. However, the increased tumour multiplicity is highly suggestive of a hereditary cancer syndrome. The absence of family history could be explained either by de novo germline events or, alternatively, by low penetrance variants.

As clinical expression of the patients included in this study could not be clearly classified as FAP or HNPPCC, we used MSI, LOH, and IHC analysis of tumour samples to establish whether the tumour exhibited either microsatellite or chromosomal instability. All tumours of the present series were MSS and immunoexpression of MMR proteins was normal for all three proteins analysed, except for two patients. In patient 5, MSH2 and MSH6 expression was negative in the carcinoma specimen and a germline missense mutation in exon 6 of MSH2 gene was found. This variant has been described both as a pathogenic mutation as well as a rare polymorphism.\(^\text{15,16}\) Although absence of MSI-H or MSI-L in colorectal tumours has been observed in patients harbouring pathogenic mutations in MSH6 or MLH1,\(^\text{17,18}\) this has not been described for patients carrying mutations in the MSH2 gene. Patient 5 clearly exhibited a colonic phenotype, which could easily be classified as an atypical polyposis, with 50 colonic adenomas distributed throughout the whole colon and a carcinoma of the splenic flexure at the age of 44. However, this was the only case of carcinoma negative for nuclear expression of β-catenin, which is rarely observed in tumours arising in the context of a germline defect of the APC gene.\(^\text{19}\) Interestingly, LOH was observed in the wild type allele at the D2S123 locus where the MSH2 gene is located. Because there is no family history, segregation analysis was not possible and functional studies of the MSH2 protein may be required in this case for further clarification. MSH6 mutation analysis will also be important to characterise this case further.

A tumour sample from patient 13 also showed absence of MSH6 expression. Despite no germline mutations being found in the MSH2 and MLH1 genes of this patient, we cannot exclude the involvement of MMR genes. Also supporting a possible involvement of the mutator pathway in this case is the lower number of adenomas compared with most patients included in this study, the absence of LOH, and the rare or absent nuclear β-catenin expression in the carcinoma and adenomas, respectively.

In the majority of tumours analysed in the present study, evidence was found for LOH and allelic imbalances at several loci. Therefore, germline mutations in the APC gene might be responsible for the florid phenotype observed in most of our patients. However, and in contrast with previous reports,\(^\text{11,12}\) we could not find any APC mutations. Pedemonte et al.\(^\text{20}\) found that five out of 18 patients with an excess of adenomas carried novel germline APC variants. However, as they stated, the pathogenicity of these variants is still uncertain though most of the patients had a clear-cut family history of CRC. Also, Frayling et al.\(^\text{21}\) studied a set of 164 patients with multiple colorectal adenomas and/or carcinomas and found that three patients, all of whom were of Ashkenazi descent, carried the I1307K variant, while four other patients had a germline E1317Q missense variant. None of these variants was found in...
the control group. Recently, Lamium et al. screened 164 unrelated patients with multiple colorectal adenomas and found that the E1317Q missense variant accounted for approximately 4% of these patients. In this study, truncating APC variants in exon 9 and in the 3’ of the gene were also found in 4/164 patients, all of them with a family history.

Considering the large number of patients included in the latter studies, it is not surprising that we did not find any of these nonsense variants or truncating mutations (5’ to exon 5, exon 9, and 3’ to codon 1580) in the admittedly limited number of patients investigated here. Our results are similar to those of Beck et al. where no APC variants were detected in a group of 25 patients who had an unusually large number of colorectal adenomas and a missense variant of a MMR gene.

In the present series, we also found that a large proportion of adenomas and carcinomas analysed showed abnormal β-catenin expression. β-catenin expression was observed in the nucleus and not in the membrane as in normal tissue, thus supporting the involvement of the Wnt pathway. A number of germline defects could result in the nuclear localisation of β-catenin in most neoplastic lesions analysed here. As mutations in β-catenin are usually dominant, it does not seem likely that germline oncogenic alterations in β-catenin would predispose to this mild phenotype. Alterations in other genes such as *ductin, axin*, and GSK3β, previously implicated in the control of β-catenin signalling to the nucleus, might also be responsible for our IHC findings. Although mutations in these genes have not been found in hereditary colorectal cancer patients, subjects with an excess of colorectal adenomas may represent a different category altogether where these genes have not been analysed yet.

According to the present analysis, we cannot exclude the presence of mutations in the APC promoter or other regulatory regions of this gene, which could affect APC expression, thereby interfering with β-catenin degradation and increasing its nuclear expression. Mutations in regulatory or non-coding regions of the APC gene are difficult to detect by standard mutational analysis.

In the present study, we also found that nuclear expression of β-catenin was more pronounced in adenomas with high grade dysplasia as compared to invasive carcinomas. This is similar to what has been described by Samowitz et al., where the percentage of somatic β-catenin mutations was significantly higher in small adenomas as compared to larger ones and invasive cancers. As these authors suggest, this might indicate that β-catenin mutations may be especially advantageous in the early stages of colorectal carcinogenesis, or that β-catenin mutations could reduce the likelihood of adenoma progression.

In conclusion, the occurrence of frequent LOH and allelic imbalances in most of the tumours included in the present series suggests that the suppressor pathway is involved in the development of multiple colorectal adenomas. The presence of nuclear expression of β-catenin in the great majority of neoplasms analysed suggests the involvement of the Wnt transduction pathway during tumorigenesis in these cases.

Further work is necessary to provide evidence for new colorectal cancer genes associated with an inherited predisposition to multiple colorectal adenomas and carcinomas. Moreover, besides genetic factors, it cannot be excluded that environmental factors, such as diet, may also contribute, in some cases, to an increased number of adenomas.

However, the fact that, as is shown in this study, the mutant pathway does not seem to be involved in the development of this phenotype further reinforces that most probably these patients do not need to be analysed for germline mutations in the MMR genes.

**ACKNOWLEDGMENTS**

We are grateful to Dr Ricardo Fodde for careful reading of this manuscript and for helpful suggestions. This research was supported by a research grant from Ministério da Saúde 1999.

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**REFERENCES**


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Cancers of the small and large bowel originate differently

A denocarcinoma of the small intestine and colorectal cancer have different genetic pathways, suggests a molecular study from Oxford. Much rarer than colorectal cancers, cancers of the small intestine are difficult to diagnose and have a poor prognosis. They tend to be adenocarcinomas and are similar to colorectal cancers. So comparing the genetic pathways of each might indicate whether the small intestine is naturally resistant to cancer or might give some clues to clinically significant differences.

Using PCR and gene sequencing on DNA extracted from paraffin sections and immunohistochemical staining sections of sections with monoclonal antibodies, the researchers looked for particular genes and proteins in the progression to colorectal cancer in 21 non-familial, non-ampullary primary adenocarcinomas of the small bowel. These included mismatch repair genes hMLH1 and hMSH2, adenomatous polyposis coli (APC) gene mutations in the mutation cluster region; and proteins β-catenin and E-cadherin at the intercellular borders that were reduced in 17 and eight of the cancers respectively, and p53 was overexpressed in the nuclei of five cancers.

One cancer resulted from replication error. Significantly, no mutations were found in the mutation cluster region of the APC gene in this relatively large study, contrasting with larger adenomas and invasive carcinomas. A beta-catenin mutation in a sporadic colorectal tumour of the RER phenotype and absence of beta-catenin germline mutations in FAP patients. Genes Chrom Cancer 1998;22:37-41.


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