Carcinogenesis in MYH-Associated Polyposis Follows a Distinct Genetic Pathway

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Abstract

Carcinogenic pathways develop according to particular genetic pathways, including the chromosomal instability (CIN+), microsatellite instability (MSI+) and MSI− CIN− routes. We have determined the genetic pathway in patients with MYH-associated polyposis (MAP), a syndrome of colorectal adenomas and cancer that results from defective base excision repair (BER). As in previous studies, MAP tumors showed a high frequency of G>T mutations in APC, in accordance with defective BER. We found that K-ras mutations were common in MAP tumors, all of the changes comprising conversion of the first guanine residue of codon 12 to thymidine (G12C, GGT>GTT). We found no BRAF mutations at the codon 599 hotspot or elsewhere in exon 14. Almost all of the MAP cancers were near-diploid (CIN−), and none was MSI+. A few p53 mutations were found, but these were not predominantly G>T changes. p53 overexpression was, however, frequent. No SMAD4 or TGFBIIR mutations were found. MAP tumors appear to follow a distinct genetic pathway, with some features of both the CIN and MSI pathways. BER deficiency is rarely accompanied by CIN or MSI. The spectrum of somatic mutations in MAP tumors reflects both selection and hypermutation to which certain guanine residues are particularly prone.

Introduction

Sporadic colorectal carcinomas develop according to particular genetic pathways (1). The most common pathway is characterized by mutations of the APC and p53 genes, by 18q allelic loss, by mutation of K-ras and SMAD4 in some cases, and by an aneuploid/polyoid/polyoid karyotype. These tumors are often said to have followed the CIN7 pathway. Other cancers are characterized by MSI aberrant DNA mismatch repair, a near-diploid karyotype, and lower levels of p53 overexpression was, however, frequent. No SMAD4 or TGFBIIR mutations were found. MAP tumors appear to follow a distinct genetic pathway, with some features of both the CIN and MSI pathways. BER deficiency is rarely accompanied by CIN or MSI. The spectrum of somatic mutations in MAP tumors reflects both selection and hypermutation to which certain guanine residues are particularly prone.

Materials and Methods

The patients with biallelic germ-line MYH were identified through Family Cancer Clinics and Polyposis Units in the United Kingdom, Finland, Denmark, and Switzerland. MYH mutation testing had been done as part of two previous studies (see Table 1). All of the patients were known to have multiple colorectal adenomas, although precise adenoma counts were not available for some patients. Most of our patient sample had developed one or more colorectal cancers, and 19 of 26 of these were available for molecular analysis.

Constitutional DNA was extracted from peripheral blood lymphocytes by standard methods. Both fresh-frozen and fixed, paraffin-embedded tumor tissues were used for the analysis, depending on their availability. DNA was extracted from the former using a standard proteinase K and phenol-chloroform method. For the latter, 5 × 10 μm unstained tumor sections were dewaxed and dissected into an appropriate amount of digestion buffer (1× magnesium-free buffer, 20 μg/ml Proteinase K) using a H&E-stained slide as a guide for the area to be microdissected.

Germline APC mutations generally lead to a phenotype of profuse colonic polyposis (FAP). One or more of these adenomatous polyps usually progresses to cancer (4), probably because of random mutations in the same genes involved in the development of sporadic colorectal cancers. It is likely, although not conclusively demonstrated, that FAP polyps can become cancerous because of progression along any of the CIN+, MSI+ or MSI− CIN− pathways (5). In HNPCC, by contrast to FAP, the phenotype is predominantly one of colorectal carcinoma (6). HNPCC is caused by germline mutations at the MSH2, MLH1, or MSH6 mismatch repair loci, and cancers in this syndrome generally follow the MSI pathway of tumorigenesis (7).

Relatively recently, a FAP-like condition has been found to result not only from germ-line APC mutations, but also from germ-line MYH mutations (8). MYH polyposis (MAP) is usually phenotypically indistinguishable from a classical or mild form of FAP, but the former is inherited as a recessive trait, with consequent implications for the risk of disease in other family members (9). MYH encodes a glycosylase, which is involved in BER and primarily targets oxidative DNA damage. In keeping with the role of MYH, colorectal tumors from MAP patients show an excess of G>T transversion mutations in the APC gene because of the failure to repair lesions induced by the variant base 8-oxo-guanine. Whereas changes in MYH expression may have some role in the pathogenesis of sporadic colorectal tumors, there is currently no evidence to show that the gene is mutated or silenced in bowel cancers outside MAP (10).

MYH is, in many ways, an unexpected gene for colorectal polyposis. Many puzzles regarding its role in tumorigenesis remain. It is not clear, for example, why germ-line MYH mutations lead to tumors of the gastrointestinal tract, or why MAP differs in its phenotype and inheritance from HNPCC. To gain additional clues to explain how MYH mutations lead to multiple colorectal tumors and cancer, we have determined the genetic pathways in 130 colorectal adenomas and 19 carcinomas from 22 MAP patients.

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The abbreviations used are: CIN, chromosomal instability; MSI, microsatellite instability; MAP, MYH-associated polyposis; BER, base excision repair; FAP, familial adenomatous polyposis; HNPCC, hereditary nonpolyposis colon cancer; F-SSCP, fluorescent single-stranded conformational polymorphism; diHPLC, denaturing high pressure liquid chromatography; LOH, loss of heterozygosity.

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For mutation analysis of APC, SMAD4, β-catenin, and p53 in tumor DNA, F-SSCP analysis was used. dHPLC was additionally used for APC. Primer pairs to amplify overlapping fragments were designed for APC codons 1200–1620 (exon 15 regions F to I). Additionally, where DNA derived from fresh-frozen tissue was available, the tumor was analyzed for APC mutations in exons 15A to 15L of APC. Primer pairs were similarly designed for the coding regions and exon-intron boundaries of p53 exons 4–8, SMAD4 (all exons and flanking regions) and β-catenin exon 3. Primer details and PCR conditions are available from the authors on request.

For F-SSCP analysis, each 25 μl PCR reaction contained 1× PCR reaction buffer without MgCl₂ (Promega), 200 μM dNTPs, 200 μM of each primer, 50 ng of genomic DNA, and 1 unit of Taq DNA polymerase (Promega). The reaction conditions are available from authors.

F-dHPLC was performed using the 3500HT WAVE nucleic acid fragment analysis system (Transgenomic, Crewe, UK) with gates being set on forward and side scatter to exclude debris, and propidium iodide signal area and width to exclude cell doublets. Propidium iodide fluorescence was collected above 670 nm, and samples were analyzed using CellQuest software.

Results

Patients (Table 1) presented at a mean age of 55 years (median, 55; range, 38–71). The mean number of adenomas in the 13 patients for whom precise counts were obtained was 70 (median, 70; range, 38–210). Adenomas were of tubular or tubulovillous morphology, with the relative ratio of normal: tumor peak areas was <0.5 or >2, thereby allowing for contamination of normal tissue within the microdissected tumor.
rectosigmoid region. The cancers had no characteristic histological pattern, with only 3 reported as showing a mucinous histology. Grade 2 cancers predominated, with 2 of 23 (9%) grade 1, 16 (70%) grade 2, and 5 (22%) grade 3. Stage varied widely among cancers (Table 1). Carcinomas were present together with adenomatous elements in many specimens.

All of the protein-truncating APC mutations in our MAP tumors were G\(\rightarrow\)T transversions. Only 1 tumor showed LOH at APC. Although the quantity of tumor tissue permitted only part of the APC gene to be screened, 22 of 105 (21%) adenomas and 6 of 14 (43%) cancers had at least one mutation (Table 2). The most commonly found changes were S1315X (9 adenomas) and E1560X (1 cancer and 11 adenomas). It is noteworthy in passing that 4 of the 19 MAP patients harbored the germ-line missense APC variant E1317Q (11); this variant, which has a population frequency of \(\sim 1\%\), is of uncertain significance, but also provided the original cause for investigation of the first MAP family reported (8).

For K-ras, the same mutation (GGT\(\rightarrow\)TGT, G12C) was found in 9 of 14 (64%) cancers and 13 of 30 (43%) adenomas tested (Fig. 2; Table 2). No other K-ras mutations were found. No BRAF mutations were found at codon 599 or in the remainder of exon 14.

We tested 14 cancers and 115 adenomas for mutations in p53. Three changes were found, all in carcinomas. One mutation, delAG-TACTGT nt380, was highly likely to be pathogenic. Two changes, P152T (C\(\rightarrow\)A, nucleotide 463) and F134V (T\(\rightarrow\)G), had been reported previously as rare changes in cancers and had potential functional importance (12). Of these three mutations, only P152T involved a
G>T change. Immunohistochemistry revealed abnormal nuclear p53 staining in 8 of 15 (53%) cancers and 2 of 41 (5%) adenomas studied. p53 mutations and immunohistochemistry were concordant (Table 2).

Seven of 15 informative cancers (47%) and 14 of 79 (18%) informative adenomas showed LOH at one or more 18q markers. We found no coding SMAD4 changes. No mutations were found in the TGFBIIR oligonucleotide tract.

None of the 146 MAP tumors showed MSI (Table 2). In keeping with this, none of 12 adenomas and 6 cancers studied showed loss of MLH1, MSH2, or MSH6 expression (details not shown). Nine adenomas (7%) and 1 cancer (6%) showed microsatellite slippage at a single marker, and 1 other cancer showed slippage at two markers. We deemed that this data did not suggest any particular tendency to MSI-low in our tumor set (13, 14). LOH was uncommon in the cancers at the markers studied (maximum of 4% of informative cases with loss at each marker, excluding the chromosome 18 microsatellites).

Consistent with the relatively low frequency of LOH, flow cytometry showed that 12 of 13 (92%) MAP cancers tested were near-diploid. The remaining cancer appeared near-tetraploid. This cancer had the characteristic K-ras mutation and 18q LOH, but no other detectable genetic changes (Table 2). To provide corroboration of the flow cytometry, 2 of the near-diploid cancers were screened for changes using comparative genomic hybridization, and no detectable changes were found (details not shown).

As expected, cancers showed a higher frequency of changes overall than adenomas (see above for details). For certain changes, such as APC mutations, this may, at least in part, have reflected the problems of studying smaller lesions using molecular methods. Methodological problems could not, however, readily explain other differences between adenomas and carcinomas, such as the more frequent aberrant expression of β-catenin and p53 in the malignant lesions. We found no other associations between clinicopathological and molecular data, including the presence of K-ras mutation (details not shown).

We compared the features of our MAP tumors with those of 107 unselected sporadic colorectal cancers studied in our laboratory. Whereas statistical comparisons must be undertaken cautiously, partly because sporadic cancers comprise a mixture of MSI+, CIN+, and MSI− CIN− lesions, our unselected series had molecular features in accordance with findings from other studies (details below) and had been analyzed using the same methods as the MAP cancers. Certain features of the MAP tumors were striking. Whereas no specific histological features distinguished the MAP tumors (details not shown), MAP carcinomas occurred at a relatively young age (median, 55; range, 38–71) compared with the sporadic group (median, 66; mean, 66; range, 27–103; χ², 15.9; P = 0.0001, Mann-Whitney test). APC mutations occurred at a similar frequency in MAP and sporadic cancers (6 of 14 versus 44 of 99; P = 0.58, Fisher’s exact test). As expected, the mutation spectrum in the APC gene was overwhelmingly biased to G>T in MAP cancers compared with the sporadic lesions; G>T changes comprised 6 of 6 truncating mutations in the MAP cancers (and 22 of 22 in MAP adenomas) compared with 44 of 44 in the sporadic cancers (P = 0.0001, Fisher’s exact test). β-Catenin mutations were very uncommon in both the MAP tumors (0%) and the sporadic cancers (1%). Nuclear β-catenin was seen in 47 of 72 sporadic and 12 of 17 MAP cancers (P = 0.46, Fisher’s exact test).

K-ras mutations were present in 9 of 14 MAP cancers (and 13 of 30 MAP adenomas) and 33 of 104 sporadic cancers (P = 0.02, Fisher’s exact test), but the spectrum in the former was restricted to the single change in codon 12, which was found in only 3 of 33 sporadic colorectal cancers (P = 0.0001, Fisher’s exact test). This comparison held true when mutation frequencies from the RASCAL study were compared with those of the MAP cancers (15). Three of 14 MAP cancers had pathogenic p53 mutations compared with 33 of 92 sporadic cancers (P = 0.23, Fisher’s exact test). Abnormal p53 overexpression was similar (P = 0.50, Fisher’s exact test) in MAP cancers (8 of 15) and sporadic lesions (26 of 53). SMAD4 mutations were not found in the MAP cancers, although such mutations were also uncommon in the sporadic cancers (P = 0.89, Fisher’s exact test). 18q LOH was as common in the MAP as the sporadic cancers (7 of 15 versus 33 of 74; P = 0.55, Fisher’s exact test). MSI was notably absent from the MAP cancers, although this was not significantly different from the 11% (12 of 107) of MSI cancers in our set of unselected colorectal carcinomas (P = 0.127, Fisher’s exact test). Diploidy was much more common in the MAP carcinomas, occurring in 12 of 13 cancers compared with 43 of 90 sporadic lesions (P = 0.002, Fisher’s exact test).

Discussion

Colorectal tumors developing in patients with biallelic germ-line MYH mutations follow a distinct genetic pathway. Although MAP is a rare, if increasingly recognized, condition and, hence, many samples are not available from MAP cancers, the data allow firm conclusions to be drawn. We have confirmed previous reports that the spectrum of somatic mutations in the APC gene largely comprises G>T transver-
sions. Mutations are present in both adenomas and carcinomas. We have found mutations to be particularly frequent at codons S1315X and at the known hotspot, E1560X, although we have not found mutations at the hotspots of codons 836 and 932 reported previously.

We have shown that the genetic instability resulting from MYH deficiency not only targets APC but also affects other genes. K-ras mutations were common in tumors from our MAP patients and were all of the same G12C (G–T) change. Previous reports from APC had suggested that MYH deficiency preferentially targeted AGAA or TGAA motifs. This motif is not present at the critical sites within K-ras, but the G–T mutations in K-ras preferentially involved the first guanine residue of codon 12 (TGTT), despite there being three other guanines within codons 12 and 13 that could have served as targets for mutation. Thus, whereas MYH mutations lead to an excess of G–T changes, the surrounding sequence affects the probability of this change, and the resulting somatic mutation spectrum reflects both selection and hypermutation.

The role of p53 in our MAP patient tumors is unclear. p53 mutations were detected in MAP cancers but, unlike APC and K-ras, there was no clear bias to G–T changes in p53. p53 protein overexpression was about as frequent in the MAP and sporadic cancers, suggesting that an alternative mechanism caused p53 overexpression in the former. SMAD4 mutation and TGFβRII mutation appear to play at most a minor role in the genesis of MAP tumors, although 18q LOH was as common as in sporadic cancers. We do not know whether or not other components of the transforming growth factor-β pathway are inactivated in MAP cancers.

All of our patient cancers were MSI−, and almost all were microdeletions (CIN−). We found no evidence of unusual levels of MSI in MAP cancers, although it has been suggested that MYH deficiency might lead to MSI-low because the MutSα complex is involved in mismatch recognition for both base excision and mismatch repair, and, hence, might be overloaded by an excess of G–T changes (16).

We conclude that the MAP pathway of carcinogenesis is distinct from both the CIN and MSI pathways, but contains elements of both. MAP tumors have high frequencies of APC mutation and low frequencies of β-catenin mutation. Like MSI+ cancers, MAP cancers tend to be near-diploid and to have low frequencies of overall LOH. Unlike sporadic MSI+ cancers, however, MAP cancers do not appear to have TGFβRII mutations. We have not determined whether or not MAP cancers harbor BAX mutations, as MSI+ cancers do, but genes such as BAX and TGFβRII are mutated in MSI+ cancers because of the presence of mononucleotide tracts (17), and we do not predict that these genes would be particularly susceptible to the G–T changes found in MAP patients. The relatively high frequencies of K-ras mutations and 18q LOH in MAP cancers more closely resemble tumors in the CIN pathway than the MSI pathway.

Overall, we hypothesize that despite overlap in the genes that are mutated, colorectal cancers generally have only one type of instability, whether CIN, MSI, or, in MAP, BER deficiency. It is not yet known whether the explanation for low levels of aneuploidy and polyplodyy in MSI+ and MAP cancers is that there is no “need” for LOH, frameshift and G–T changes, respectively, occurring at sufficiently high frequencies, or that too much genetic instability harms the cell. For MSI+ cancers, the subsequent genetic pathways can partly be explained because certain genes with short repetitive sequences are susceptible to mutation (although this cannot easily explain the higher frequencies of β-catenin and BRAF mutations in these tumors). There is currently no good evidence to show that certain genes are especially prone to the incorporation of 8-oxo-guanine or to resistance to repair of an 8-oxoG:A mismatch, although certain sequences are preferentially mutated in MAP tumors. The possibility remains that MAP cancers resemble MSI+ cancers in their gross genetic features such as karyotype, but are more like CIN+ cancers in the genes that are mutated.

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References


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