Chromosomal Instability in MYH- and APC-Mutant Adenomatous Polyps

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Abstract

The vast majority of colorectal cancers display genetic instability, either in the chromosomal instability (CIN) or microsatellite instability (MIN) forms. Although CIN tumors are per definition aneuploid, MIN colorectal cancers, caused by loss of mismatch repair function, are usually near diploid. Recently, biallelic germ line mutations in the MYH gene were found to be responsible for MYH-associated polyposis (MAP), an autosomal recessive predisposition to multiple colorectal polyps, often indistinguishable from the dominant familial adenomatous polyposis (FAP) syndrome caused by inherited APC mutations. Here, we analyzed MYH- and APC-mutant polyps by combining laser capture microdissection, isothermal genomic DNA amplification, and array comparative genomic hybridization. Smoothed quantile regression methods were applied to the MAP and FAP genomic profiles to discriminate chromosomes predominately affected by gains and losses. Up to 80% and 60% of the MAP and FAP polyps showed aneuploid changes, respectively. Both MAP and FAP adenomas were characterized by frequent losses at chromosome 1p, 17, 19, and 22 and gains affecting chromosomes 7 and 13. The aneuploid changes detected at early stages of MYH-driven tumorigenesis may underlie accelerated tumor progression, increased cancer risk, and poor prognosis in MAP. (Cancer Res 2006; 66(5): 2514-9)

Introduction

The vast majority of colorectal cancers display genomic instability, either in the chromosomal instability (CIN; invariably aneuploid) or microsatellite instability (MIN/MSI; mostly near diploid) forms (1, 2). Although MIN and CIN were initially reported as mutually exclusive, in a subset of colorectal cancer, neither CIN nor MIN are found (3). Aneuploidy is often thought to account for the malignant potential and poor prognosis characteristic of CIN tumors. CIN, usually defined as an accelerated rate of chromosome missegregation during cell division, is thought to result from mitotic defects at checkpoint genes (1, 4). In addition, genetic inactivation of the APC tumor suppressor gene has been shown to cause both tetraploidy and aneuploidy in primary mouse cell lines (5, 6). However, the molecular basis of CIN is to date still largely unknown: mutations in checkpoint genes and/or other CIN candidate genes have been found at only relatively low incidences (4, 7, 8), and APC-mutant polyps do not show major aneuploid changes (9), although the latter is contrasted by other studies showing allelic imbalances in early colonic adenomas (4, 10).

Recently, biallelic germ line mutations in the MYH gene, a gene coding for a DNA glycosylase involved in the base excision repair (BER) pathway, were found to cause MYH-associated polyposis (MAP), an autosomal recessive polyposis syndrome often indistinguishable in its clinical manifestations from classic or attenuated forms of familial adenomatous polyposis (FAP; ref. 11). Clinical presentation of symptomatic MAP is usually around 50 years of age and, although a spectrum of MAP extracolon manifestations has not been defined yet, accumulating literature suggests that MYH mutations may also confer increased risk for other tumor types (12-17).

BER genetic instability is marked by G:C to T:A transitions, which is in agreement with the reports that colon cancer–related genes like APC and KRAS2 display an excess of these somatic mutations in MAP tumors (11, 18, 19). This form of genetic instability is distinct from MSI, which is apparently absent among MAP tumors (20). In the latter study, MAP tumors analyzed by flow cytometry and conventional loss of heterozygosity (LOH) displayed near-diploid karyotypes and a low incidence of LOH (20). Here, we have evaluated the CIN status of MYH- and APC-mutant polyps by array comparative genomic hybridization (CGH) on bacterial artificial chromosomes (BAC) to define MAP- and FAP-specific genomic signatures.

Materials and Methods

Patient specimens were obtained from the Department of Surgery, Heinrich Heine University, Germany; the Institute of Medical Genetics, Cardiff University, United Kingdom; the Department of Pathology, Leiden University Medical Center; the Department of Surgery, Erasmus Medical Center, the Netherlands; and from the Department of Surgery, Erasmus Medical Center, the Netherlands. A total of 12 baseline control samples (nine diluted DNA controls and three DNA samples from normal epithelial microdissected mucosa from healthy individuals; ref. 21) were also included to control for bias introduced by the downstream DNA amplification.

Sample processing procedures, including laser capture microdissection (LCM) of 1,000 parenchymal cells (600,000 μm² area), DNA extraction, and φ29 amplification, were done as previously described (21). To avoid bias in sampling small areas of the tumors otherwise not representative of the entire neoplasia, we have done LCM by collecting several independent areas of each polyp. In addition to array production and testing using the human 3600 BAC/PAC genomic clone set from the Welcome Trust Sanger Institute,9 sample labeling and hybridization procedures are also fully referenced elsewhere (21).

Data analysis was done as previously described (21) using a set of functions implemented in R (22)10 with few modifications. Briefly, a
GenePix data file (.gpr) was generated per array using the software GenePix Pro 4.0 (Axon Instruments, Union City, CA). Each .gpr file was directly loaded into the R environment using the marrayTools package to extract the background corrected Cy3 and Cy5 median raw intensity per spot. Intensity data were normalized with the variance stabilization and normalization function implemented in the vsn package (23). The resulting normalized intensities were transformed into log 2 scale. Cy3 and Cy5 median intensities were subsequently extracted from each set of triplicate spots to produce a unique value per BAC clone. A log 2 Cy3/Cy5 ratio was thereafter calculated. The final data set, used in subsequent analysis, consisted of 3,397 independent observations per sample. For all experiments, data were organized per chromosome, and clones were

![Figure 1](image.png)

**Figure 1.** Example of smoothed median profile array CGH analysis (chromosome 19) of a normal and dysplastic intestinal sample. Standard H&E staining of 10-μm sections from normal colonic epithelium (A) and dysplastic colonic crypts from a low-grade dysplasia adenoma (B). Smoothed medians of copy number changes from normal (C) and dysplastic (D) microdissected colonic crypts, using chromosome 19 as an example for graphical visualization and calculated with the array CGH data smoothing algorithm recently implemented in R (24). For each chromosome, the smoothed median curve of each sample (red) was compared with the median curve of the 12 baseline controls (green), to facilitate the detection of loss/gain events often masked by the occasional variability of individual genomic clones (small open circles) introduced by the q29 whole-genome amplification procedure. Upper and lower boundaries for data dispersion (blue lines; quantiles of order 15% and 85%) were also computed for each individual sample by applying the same method. It is expected that 70% of the data points for each sample are contained within the quantile boundaries. A loss/gain event was scored (see also Supplementary Data) as shown in (D); that is, whenever the smoothed medians (green line) lay outside the upper and lower quantile sample boundaries (blue lines) and clearly distinct from the sample smoothed median curve (red line). Whenever both baseline and sample smoothed medians were contained within the quantile boundaries as shown in the normal sample (C), no loss/gain event was scored.

### Table 1. Patient and sample information

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender/age of onset</th>
<th>Germ line mutation</th>
<th>Colorectal carcinoma</th>
<th>No. polyps, total analyzed*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MAP samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D8</td>
<td>M/60</td>
<td>G38D/G382D</td>
<td>No</td>
<td>62</td>
</tr>
<tr>
<td>RE1</td>
<td>M/46</td>
<td>Y165C/G382D</td>
<td>1 cecum</td>
<td>&lt;100 microscopic</td>
</tr>
<tr>
<td>UK1</td>
<td>M/50</td>
<td>Y165C/G382D</td>
<td>1 (intramucosal)</td>
<td>40</td>
</tr>
<tr>
<td>UK2</td>
<td>F/51</td>
<td>Y165C/G382D</td>
<td>No</td>
<td>50</td>
</tr>
<tr>
<td>UK3</td>
<td>M/45</td>
<td>Y165C/Y165C</td>
<td>No</td>
<td>&gt;100</td>
</tr>
<tr>
<td><strong>FAP samples</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D1</td>
<td>M/43</td>
<td>Exon 15 del c1061 (AAAAC)</td>
<td>No</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D2</td>
<td>F/13</td>
<td>Exon 15 del c1061 (AAAAC)</td>
<td>No</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>D5</td>
<td>M/30</td>
<td>Exon 15 c1035 (C &gt; T)</td>
<td>No</td>
<td>&gt;100-1,000</td>
</tr>
<tr>
<td>D9</td>
<td>M/35</td>
<td>Exon 13 c563 (del T)</td>
<td>No</td>
<td>&gt;100</td>
</tr>
<tr>
<td>D12</td>
<td>F/22</td>
<td>Exon 15 1</td>
<td>No</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>D15</td>
<td>F/23</td>
<td>Exon 15 c1035 (C &gt; T)</td>
<td>No</td>
<td>100-1,000</td>
</tr>
<tr>
<td>D18</td>
<td>ND</td>
<td>Exon 9 ins1102</td>
<td>No</td>
<td>100-500</td>
</tr>
<tr>
<td>L2</td>
<td>M/58</td>
<td>Exon 11 c316 (G &gt; C)</td>
<td>No</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Analyzed samples included laser capture microdissected samples from low-grade dysplastic areas (T) and apparently normal areas (N).

1Mutation detected by protein truncation test but not yet confirmed by sequencing.
ordered from chromosome 1 to chromosome Y according to their Golden Path Mb position.11

Data were analyzed separately for each chromosome with the array CGH data smoothing algorithm recently implemented in R (24). In brief, a L1 quantile regression method that uses BAC clones spatial information (Mb position) was first applied to log 2 normalized ratios to calculate the smoothed median of multiple subsequent BAC probes. Second, upper and lower bounds for data dispersion were also computed for each sample using the same method but now based upon quantiles of order 15% and 85%. It is expected that 70% of sample data points are contained within the quantile bounds. Smoothing was done using a $k$ smoothing variable of 4 for all calculations. Due to the overall data dispersion introduced by the whole-genome amplification method, the 12 control samples described elsewhere (21) were used to estimate and test the amount of smoothing needed. Median log 2 ratios per clone of these control samples was calculated to create an “averaged” amplified normal array CGH baseline profile and the smoothed medians, 15% and 85% upper and lower quantile bounds, were also calculated. Per chromosome, the smoothed median of each normal or tumor sample was compared with the median trend of the baseline controls (Fig. 1), which facilitates detection of data trends, clearly discernible from the variation introduced by the $\Phi 29$ whole genome amplification procedure. Data was scored as a loss/gain event (Supplementary Data) when the baseline smoothed medians were positioned outside the upper and lower quantile sample bounds. A 5-Mb region was the minimal region scored.

Results and Discussion

CIN analysis is often hampered by technical and sample limitations. Specific CIN patterns associated with small but clinically relevant tumor areas may pass undetected due to intratumor heterogeneity and/or the presence of contaminating normal cells. Thus, we have investigated the ploidy status of MAP and FAP polyps by combining LCM, isothermal genomic DNA amplification, and array...
CGH; a highly sensitive and quantitative method is ideally required to detect chromosomal aberrations in small numbers of microdissected tumor cells, as previously developed and validated (21).

A total of 80 laser capture microdissected samples (55 FAP and 25 MAP) were selected from snap-frozen adenomatous intestinal polyps with intraepithelial neoplasia and from the corresponding normal epithelial intestinal mucosa (22 normal and 58 dysplastic samples). Samples were collected from unrelated MAP ($n=5$) and FAP ($n=8$) patients (MAP mean age = 50.4 years; range, 45-60 years; FAP mean age = 32 years; range, 13-58 years) carrying biallelic MYH and monoallelic APC germ line mutations, respectively (Table 1).

Among the MAP patients, three were compound heterozygous for Y165C and G382D, the two most common MYH missense changes among MAP Caucasian patients (13, 14); one was homozygous for Y165C and G382D. Among the FAP patients, five carried a truncating germ line mutation in exon 15, whereas the three remaining patients had mutations located in exons 9, 11, and 13. Among MAP patients, an average of 70.4 polyps (range, 40 to >100) were found. FAP patients had on average >100 polyps. Two to six polyps were analyzed for each individual patient (Table 1). In addition, all samples were matched as far as histology (low-grade dysplasia). Accordingly, we could not detect any somatic KRAS mutation (exons 12 and 13), thus confirming the early stage of the analyzed polyps (data not shown).

Epithelial colonic cells were microdissected by LCM from normal and dysplastic areas (Fig. 1). Visualization and scoring of chromosome imbalances present in the analyzed adenomas was done by means of the nonparametric tool "array CGH data smoother" via quantile regression (ref. 24; Fig. 1). Among the APC-mutant polyps, a total of 150 events affecting complete chromosome arms were counted (2.7 average events per sample), whereas a total of 147 events were counted among the MAP polyps (5.9 average events per sample) using the array CGH smoother. However, when smaller regions encompassing multiple neighboring BAC clones [e.g., the chromosome 1p subtelomeric region (tel-50Mb)] are also included in the analysis, gain/loss events were observed in 23 of 25 (92%) and 29 of 55 (53%) MAP and FAP samples, respectively, adding up to a total of 336 and 446 genomic imbalance events among MAP and FAP polyps, respectively (average events per sample 13.4 and 8.2, respectively; Supplementary Data).

Unsupervised one-dimensional hierarchical clustering analysis of smoothed medians array CGH data from MAP and FAP samples (Fig. 2) shows that there is no clear-cut resolution of the MAP and FAP samples, which is consistent with the qualitative similarity of the observed aneuploidy changes between the two groups of polyps. All FAP- and MAP-specific gains and losses were scored (Supplementary Data) and plotted as shown in Fig. 3. Loss events occurred more frequently than gains in both groups, and, overall, the most prevalent aberrations were gains of chromosome 7 (9% and 20%) and chromosome 13 (3.6% and 28%), and losses of whole chromosome 17p (27.2% and 64%), chromosome 19p (58% and 84%), and chromosome 22q (43.6% and 84%) among FAP and MAP samples, respectively. Loss of 5q was detected only in few samples as also previously reported in ref. (21). The latter is in line with expectations as it has been extensively reported that somatic APC point mutations represent a frequent second-hit mechanism in FAP. Notably, some of the samples histologically classified as normal intestinal epithelia but microdissected from an area in direct proximity of a dysplastic area frequently displayed the same trend in CIN patterns as the corresponding neoplastic neighboring area, both for FAP (e.g., D5_8D and D5_8H; Fig. 2) and MAP (e.g., UK2_4 and UK2_4N; Fig. 2) polyps. The microdissected normal control samples (CON) did not display any detectable genomic abnormality (Fig. 2). Our results show that a considerable degree of aneuploidy changes is already detectable at very early stages of adenoma progression in both FAP and MAP. The patterns of chromosomal regions affected by gain and loss events of FAP and MAP polyps are partly overlapping. Notably, MYH-mutant polyps show a twice higher overall incidence of aneuploidy when compared with FAP adenomas (ratio of 2.2 for complete chromosome arms and of 1.7

Figure 3. Percentage of chromosomal aberrations in MYH- and APC-mutant genomes. Chromosomal regions affected by gains and losses were scored as described in Fig. 1. Complete or regional chromosome arms gains/losses were counted separately (Supplementary Data), and the percentage of events was calculated per chromosome.
for all genomic events). A paired t test was used to assess the statistical significance of such differences. The results showed that these differences are statistically significant at 95% confidence level \((P = 0.002\) and \(P = 0.033\) for complete chromosome arm losses and gains, respectively; \(P = 0.0005\) and \(P = 0.0099\) for all loss and gain events, respectively). Furthermore, the results of the paired t test analysis also show that the genomic events are not randomly distributed and point to the presence of a clear trend in the affected chromosomal regions of both FAP and MAP polyps.

These findings contrast with those reported by the Lipton et al. study (20), where flow cytometry analysis of 13 MAP tumors revealed near-diploid and near-tetraploid genomic contents with no detectable aneuploid changes. Several explanations may account for these apparent discrepancies. First, flow cytometry is the traditional method for analysis of ploidy status of tumor samples. The degree of DNA ploidy (DNA index or DI) is evaluated according to preestablished criteria, and samples are classified as diploid or aneuploid according to specific histogram patterns. However, aneuploid changes below specific thresholds are likely to go undetected by flow cytometry. In the present study, a more sensitive and specific tool was employed (i.e., array CGH) that allows to measure relative DNA abundances with a sensitivity that theoretically ranges from abnormalities affecting complete chromosome arms to a few megabases. On the other hand, this technology is unable to detect balanced quantitative abnormalities, such as tetraploidy. Accordingly, other studies revealed frequent genomic abnormalities by conventional CGH analysis in sporadic colorectal cancers previously classified as near diploid by flow cytometry (3). A second explanation for the discrepancy between our results and the study by Lipton et al. may reside in the tumor sampling procedure [i.e., the use of laser capture microdissected parenchymal cells (this study) versus whole tumor samples (20)]. LCM allows the study of intratumor heterogeneity (e.g., pure populations of normal versus hyperplastic versus dysplastic colonic cells) and also avoids sample contamination with infiltrating normal cells from the tumor microenvironment (e.g., fibroblasts, endothelial cells, and lymphocytes). In a previous study of CIN in early sporadic adenomatous polyps and fully progressed carcinomas by inter-simple sequence repeat PCR, Stoler et al. could detect a significantly higher genomic instability in carcinomas compared with adenomas only when using laser capture microdissected specimens (25). Finally, our observation that a low but considerable degree of aneuploidy characterizes early stages of colorectal tumor progression, although in apparent disagreement with some reports (9, 20), is corroborated by others showing clear allelic imbalances in early colonic adenomas (4, 10).

The identification of aneuploidy at early stages of tumor formation in MYH- and APC-mutant polyps is interesting also in view of previous reports showing that loss of APC function in primary mouse cell lines results in CIN due to a kinetochore attachment defect at mitosis (5, 6). It is generally accepted that APC’s main tumor suppressing activity resides in its capacity to bind and regulate Wnt/β-catenin signal transduction (2). However, additional APC functions in cytoskeletal organization, mitotic spindle assembly, cell migration, and apoptosis may play important roles in tumor progression and malignant transformation (2, 26). The relatively low CIN levels here observed in FAP polyps from APC mutation carriers may be explained by the fact that loss of APC function is necessary but not sufficient to trigger full-blown CIN in adenomas. The CIN defect caused by APC mutation is likely to be subtle, and additional somatic alterations in other genes may act synergistically with APC to enhance CIN. However, our observation of aneuploid changes in apparently normal mucosa specimens adjacent to polyposis lesions indicates that CIN may even precede adenoma onset in polyposis patients. In MAP, polypl formation is likely to be triggered by G:C to T:A transversions in the APC gene resulting from the mutator phenotype due to loss of BER function (11, 18). However, other genes, including those that act synergistically with APC in triggering CIN, are expected to undergo increased somatic mutations in the BER-deficient cellular environment, thereby resulting in the relatively higher incidence of aneuploid changes observed in MAP when compared with FAP polyps.

The observation that CIN is already detectable at early stages in MYH-mutant tumors may underlie accelerated tumor progression and poor prognosis in MAP. Notably, a recent study on Dutch MAP patients has indeed shown that clinical features such as polypl multiplicity, colorectal cancer risk, and extracolonic manifestations are more severe than previously reported and often indistinguishable from those of FAP patients carrying APC germ line mutations (17). Future studies with enlarged polypl cohorts analyzed by expression and genomic profiling and by somatic mutation analysis will pinpoint the genes and the corresponding signaling pathways selectively altered in MAP and FAP adenomas and their relevance for progression towards malignancy.

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