Human Cancer Biology

Chromosome 5q Loss in Colorectal Flat Adenomas

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Abstract

**Purpose:** Flat adenomas are a subgroup of colorectal adenomas that have been associated with a more aggressive clinical behavior compared with their polypoid counterparts. Here, we aimed to compare one of the molecular changes most explicitly associated with adenoma to carcinoma progression, that is, chromosomal instability, between flat and polypoid colorectal adenomas.

**Experimental Design:** Consecutive series of 83 flat and 35 polypoid adenomas were analyzed for DNA copy number changes using a high-resolution array comparative genomic hybridization platform, microsatellite instability (MSI) status, and for mutations in the adenomatous polyposis coli (APC) gene. Immunohistochemical stainings for CD3, CD8, and FoxP3 expression were carried out.

**Results:** Patterns of DNA copy number changes differed between the two phenotypes, with significantly more frequent loss of 5q14.3 and 5q15-q31.1 in flat adenomas, whereas losses of 1p36.32-p35.3, 10q25.3, 17p12, and chromosome 18 were more frequent in polypoid adenomas (false discovery rate < 0.2). MSI was observed in one flat adenoma. As the 5q15-q31.1 region harbors the APC locus, APC mutation status was investigated, showing significantly less mutations in flat adenomas ($P = 0.04$). An initial exploration of a possible association of 5q loss with inflammation indicated that tumor-infiltrating lymphocytes were more abundant in the stroma of flat adenomas compared with that of polypoid adenomas.

**Conclusion:** Flat and polypoid adenomas have partially distinct chromosomal profiles, consistent with differences in the biology underlying these phenotypes. Alterations more specific to flat adenomas, in particular 5q loss, may be associated with inflammation. *Clin Cancer Res; 1–10.* ©2012 AACR.

Introduction

Colorectal cancer (CRC) is caused by an accumulation of alterations in the (epi)genome of the epithelial cells that line the large intestine, first giving rise to an adenoma, that in a minority of cases can progress into an invasive, metastasizing adenocarcinoma. Next to the well-known polypoid adenoma, Muto and colleagues showed in 1985 the existence of flat adenomas in the large intestine (1). Current paradigms of multistep colorectal carcinogenesis are largely based on the "polyp cancer sequence," that was coined by Morson in 1974 and for which molecular basis was provided by Vogelstein and colleagues (2, 3). In this sequence, the terms "polyp" and "adenoma" have unrightfully been used as synonyms, thereby largely ignoring the notion that nonpolypoid precursors of sporadic CRC could exist. Subsequently, observations made in Japan of the undisputable existence of flat adenomas were long interpreted as reflecting a non-Western entity. Recent studies, however, using advanced endoscopic imaging techniques, have reported similar incidences of flat tumors in western countries to those in the East, ranging from 6.8% to 36% (4–6). For the GI community at large, this has changed only recently with publications on large U.S.-based series of flat adenomas (6).

For obvious reasons, flat adenomas can more easily go undetected during colonoscopy, especially when bowel preparation is suboptimal or the cecum, where flat adenomas occur relatively frequent, has not been fully inspected. Consequently, flat adenomas have been suggested as an important cause of interval cancers in screening programs.
Translational Relevance

Flat lesions are a distinct type of colorectal lesions representing a substantial part of all colorectal cancer (CRC) lesions. They are associated with a more aggressive clinical behavior compared with their polypoid counterparts. At present little is known about the genomic alterations present in flat lesions.

This study shows that flat adenomas harbor significantly more chromosome 5q loss compared with polypoid adenomas and at the same time they show significantly less APC truncation mutations. These 2 molecular characteristics are also observed in colitis-associated CRC, potentially suggesting parallels in the genesis/development of flat lesions and colitis-associated CRC. The loss of 5q has previously been associated with metastasis and as such can be considered a marker for aggressive lesions. This knowledge contributes to better classification of flat lesions based on molecular features, which will result in the identification of flat adenoma patients that may need closer follow-up.

Moreover, it has been suggested that these lesions would complete the adenoma to carcinoma progression at higher speed, adding to the risk of interval cancers (6, 7). Another issue relates to the potentially higher progression risk of flat adenomas as visualized in Supplementary Fig. S1 (6). Moreover, it has been suggested that these lesions would complete the adenoma to carcinoma progression at higher speed, adding to the risk of interval cancers (6, 7).

Another issue relates to the potentially higher progression risk of flat adenomas as visualized in Supplementary Fig. S1 (6, 8). Formal estimations of progression risk would require unethical longitudinal studies leaving adenomas untreated. However, it is feasible to determine the biologic features underlying this progression. Initial molecular studies indicated a different tumor biology for flat lesions, including a lower incidence of KRAS mutations (9, 10), but recent studies contradict these findings (11). This controversy may be due to methodologic issues including small sample sizes, heterogeneous definition of flat adenomas, and selection bias. Consequently, much of the genomics of flat colorectal adenomas remains to be elucidated.

In this context, genomic instability is of particular interest as it plays a crucial role in the pathogenesis of CRC, especially at the stage of adenoma to carcinoma progression. Genomic instability in CRC occurs in about 15% of colorectal carcinomas through the microsatellite instability (MSI) pathway, in which instability occurs at the nucleotide level resulting in the accumulation of multiple mutations (12). The more common pathway is the chromosomal instability pathway (CIN), marked by DNA copy number alterations and structural rearrangements (13). Interestingly, specific patterns of DNA copy number changes have been associated with adenoma to carcinoma progression (14), making this molecular feature particularly interesting to compare between these 2 adenoma phenotypes. Therefore, the aim of this study was to compare DNA copy number aberrations between flat and polypoid adenomas in a well-defined series using a high-resolution array comparative genomic hybridization (aCGH) platform.

Materials and Methods

Patient and sample selection

Formaldehyde-fixed, paraffin-embedded (FFPE) polypoid and flat colorectal adenoma tissue samples were consecutively collected at 5 different institutes. Exclusion criteria were patients with hereditary forms of CRC [including familial adenomatous polyposis (FAP) and hyperplastic polyposis syndrome], inflammatory bowel disease (IBD), hyperplastic polyps, adenoma size smaller than 5 mm (except for 2 depressed adenomas, subtype 0-Ic, as these were of clinical interest) and insufficient DNA quality, resulting in a series of 83 flat adenomas and 35 polypoid adenomas. All flat lesions were detected by selective chromoendoscopy (dye-spraying), which has been suggested to enhance the detection rate of these lesions (15). Of these 118 cases, 37 adenomas from 32 patients were from Leeds General Infirmary, Leeds, United Kingdom, collected between 1995 and 2007 (5), 17 adenomas from 16 patients were from Hospital Vitkovice, Ostrava, Czech Republic, collected between 2006 and 2008 (16), 17 adenomas from 17 patients were from Maastricht University Medical Center, Maastricht, The Netherlands, collected between 2008 and 2009, 12 adenomas from 11 patients were from Tokyo Metropolitan Geriatric Hospital, Tokyo, Japan, collected between 2005 and 2006 and 35 adenomas from 34 patients were from the VU University medical center, Amsterdam, The Netherlands, collected between 2006 and 2008. Archival material was used in compliance with the institutional ethical regulations for use of patient material and national guidelines. Histologically flat adenomas are usually defined as lesions whose dysplastic mucosa is not more than twice that of the surrounding normal mucosa. This method, however, is not always very accurate (17), and in line with current conventions in literature (4–7, 18), this study used the endoscopic Paris classification (19). Five different categories of adenomas were discerned; pedunculated, 0-Ip (n = 35), slightly elevated, 0-IIa (n = 48), slightly depressed 0-Iic (n = 3), lateral spreading type flat, LST-F (n = 23), and the lateral spreading type granular, LST-G (n = 9), in which LST is a subclass of the Ila type and defined as being larger than 10 mm (20). A summary of all clinical characteristics, as recorded by the GI endoscopists, is listed in Table 1. Histologic information was obtained by revising all hematoxylin and eosin (H&E)-stained slides by one pathologist according to European guidelines (18) to avoid potential interobserver bias (Table 1). Major histologic types observed concern traditional flat adenomas with dysplasia of intestinal crypts similar to that seen in tubular adenomas and flat adenomas with saw tooth appearance of the epithelium lining the crypts with often no evident dysplasia (Supplementary Fig. S1).

DNA isolation

DNA from FFPE material was isolated following macrodissection (>70% dysplastic cells), as described before (21), with a few modifications. A 5-day incubation period with lysis buffer (ATL buffer, QiAmp, DNA micro-kit, Qiagen)
and freshly added (once every day) proteinase K (10 pmol per mL) was carried out. DNA was isolated with the QiAmp DNA micro-kit (Qiagen) and concentrations and purity was measured on a Nanodrop ND-1000 spectrophotometer (Isogen).

### DNA quality control and aCGH

DNA quality was tested using the CGH Labeling Kit for Oligo Arrays (Enzo Life Science) in which a specific activity (pmol per mL dye/μg per μL genomic DNA) lower than 30 pmol/μg was considered insufficient and excluded for aCGH.

Hybridizations were carried out using arrays that contained 180,080 in situ synthesized 60-mer oligonucleotides (4×180K, GPL8687; Agilent Technologies), representing 169,793 unique chromosomal locations evenly distributed across the genome (space ~17 kb) and 4,548 additional unique oligonucleotides located at 238 of the Cancer Census genes.

Labeling, hybridization, scanning, and feature extraction were carried out as previously described for the 4×44K Agilent array (22). Microarray scanner G2505B (Agilent technologies) was used for scanning and feature extraction software (version 10.5, Agilent Technologies, protocol CGH_105_Dec08) was applied using default settings. To keep all experiments comparable, no quality flagging was applied, and all oligonucleotides were included in the downstream analysis.

Hybridizations were done according to the across aCGH approach as described before (22). As reference samples, normal DNA from resection margins of 11 male or 11 female noncancer patients were used. Data are made publicly available in GEO (www.ncbi.nlm.nih.gov/projects/geo), accession number GSE30479.

### Array CGH analysis

The probes were positioned along the genome according to the NCBI36/hg18 build (March 2006). Log2 ratios were normalized by subtraction of the median value of all the probes spotted on the array. To avoid potential wave bias, a wave-smoothing algorithm was applied (23).

Segmentation and calling of the data was done using CGHCall (version 2.5) with SD-Undo of 2 and SD-Undo-long of 3. Segments with a probability score of more than 0.5 were considered gained, amplified, or lost (24).

Due to the use of a common reference pool, it could not be excluded that small focal aberrations found could in fact be germ line copy number variations (CNV). Due to the fact that these samples originate from different ethnic backgrounds, this could potentially result in identification of significantly different CNVs unrelated to the phenotype differences we are interested in. To avoid this potential bias, CNVs were identified and discarded from the downstream analyses as follows. Briefly, CNV segments were found by plotting in silico aCGH results from 219 FFPE normal colon mucosa samples against the normal male and female pools that were used in this study. All aberrations that were smaller than 3MB and that were called by the calling algorithm CGHCall were considered as CNV segments and therefore these loci were removed from the analysis. In this way, 23,268 probes were removed, leaving 151,073 probes to be analyzed. Of the removed probes, 98.5% was present gain, amplified, or lost.

DNA instability status

MSI analysis was done using the MSI Analysis System (MSI Multiplex System Version 1.2; Promega) consisting of 5 quasimonomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, and MONO-27) according to

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**Table 1. Clinical characteristics of the 118 adenomas used for aCGH analysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Polyoid adenomas</th>
<th>Flat adenomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 35)</td>
<td>(n = 83)</td>
<td></td>
</tr>
<tr>
<td>Total no. of patients</td>
<td>34</td>
<td>77</td>
</tr>
<tr>
<td>No. of patients with One lesion</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>&gt;One lesions</td>
<td>8 (2–5)</td>
<td>44 (2–9)</td>
</tr>
<tr>
<td>Carcinoma present</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Metastasis present</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Male/Female</td>
<td>18/17</td>
<td>54/29</td>
</tr>
<tr>
<td>Age (y)</td>
<td>71.7 (53–89)</td>
<td>69.3 (28–90)</td>
</tr>
<tr>
<td>Location</td>
<td>Proximal</td>
<td>5</td>
</tr>
<tr>
<td>Distal</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Rectum</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Size (mm)</td>
<td>19 (6–64)</td>
<td>18 (3–100)</td>
</tr>
<tr>
<td>Paris classification</td>
<td>0-Ip</td>
<td>35</td>
</tr>
<tr>
<td>0-IIa</td>
<td>48</td>
<td></td>
</tr>
<tr>
<td>0-IIc</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>LST-G</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>LST-F</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>Tubular</td>
<td>19</td>
</tr>
<tr>
<td>Tubulovillous</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Villous</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Serrated</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Dysplasia</td>
<td>LGD (mild)</td>
<td>1</td>
</tr>
<tr>
<td>LGD (moderate)</td>
<td>30</td>
<td>64</td>
</tr>
<tr>
<td>HGD (severe)</td>
<td>4</td>
<td>14</td>
</tr>
</tbody>
</table>

NOTE: Patients with familial forms of CRC, IBD, or hyperplastic polyps were excluded.
manufacturer’s instructions. PCR products were separated using a 3500 Genetic Analyzer (Applied Biosystems) and analyzed using GeneScan 3100 (Applied Biosystems). An internal lane size standard was added to the PCR samples for accurate sizing of alleles and to adjust for run-to-run variations. When 2 or more markers were instable, the sample was interpreted as microsatellite unstable (MSI); all other samples were classified as microsatellite stable (MSS).

**APC mutation analysis**

The APC mutation cluster region (MCR1286-1513) contains 65% of all known somatic mutations of APC (28). Mutation status was analyzed by sequencing the MCR between codons 1260 and 1530 by 4 flanking PCRs followed by 2 seminested PCRs, as described (29). Sequencing was done on a 3500 Genetic Analyzer (Applied Biosystems) and analyses were carried out with VectorNTI (Invitrogen) and Mutation Surveyor (SoftGenetics). Mutations were reconfirmed by independent PCR reactions and sequencing.

**Triple fluorescent immunostaining of tumor-infiltrating lymphocytes**

Simultaneous immunohistochemical staining of 3 different epitopes was applied to 4-μm FFPE sections of 9 polyoid adenomas and 12 flat adenomas (7 with a 5q loss and 5 without a 5q loss), as described before (30), using the following primary antibodies and fluorescent antibody conjugates: ab828 (rabbit polyclonal, anti-CD3; Abcam), 4B11 (mouse monoclonal IgG2b, anti-CD8; Novocastra), clone 236A/E7 (mouse monoclonal, anti-FoxP3 antibody (IgG1, Abcam), goat anti-rabbit IgG-Alexa Fluor 488, goat anti-mouse IgG2a-Alexa Fluor 488, and goat anti-mouse IgG1-Alexa Fluor 647 (Molecular Probes)]. Images were captured with a confocal laser scanning microscope (LSM510; Zeiss) in a multitrack setting. These specific subsets of cytotoxic (CD3+CD8+) and regulatory T cells (Tregs, FOXP3+) are indicators of host immune response. Samples were scored for high, intermediate, or low expression of tumor-infiltrating lymphocytes (TIL). One representative image was scanned per sample.

**Statistical analysis**

For unsupervised data analysis, hierarchical cluster analysis using Weighted Clustering of Called aCGH data (WECCA) was carried out (27).

A binomial test on differential proportions of alterations was carried out (CGHTest; www.few.vu.nl/~mavdwiel). This test procedure includes a permutation-based false discovery rate (FDR) correction for multiple testing. Alterations occurring in less than 5% were a priori excluded and an FDR < 0.2 was considered statistically significant. Differences in frequencies of APC mutation or size between flat and polyoid lesions were evaluated by χ² test.

**Gene ontology analysis**

To interpret the biologic relevance of the genes located at the altered chromosomal regions, a gene ontology analysis was carried out (Ingenuity Systems). Biologic/molecular functions were considered to be significantly overrepresented when they contained more than 20 genes and the Benjamini–Hochberg corrected P was less than 0.1.

**Results**

**DNA copy number changes**

In this study, we compared high-resolution genome-wide chromosomal profiles of a well-defined series of flat and polyoid colorectal adenomas. Overall the adenoma size was similar between the 2 groups (P = 0.4). To determine common chromosomal alterations, the frequencies of gains and losses per probe were plotted for flat and polyoid adenomas separately (Fig. 1). Overall, both phenotypes showed low frequencies of DNA copy number aberrations. Forty-eight of the 83 flat adenomas (58%) and 22 of 35 polyoid adenomas (63%) had DNA copy number aberrations for more than 1% of 151,073 probes. On average, flat adenomas showed 3.07 alterations (range 0–19) per sample, with a mean number of 1.61 gains (range 0–10) and 1.46 losses (range 0–15). Frequent alterations (occurring in >10% of the cases) included gains on chromosome 7p22.1-q36.3, 13q12.11-q34, and 20p13-q13.33 and losses on 5q15-23.2. Polyoid adenomas showed 4.14 alterations on average per sample (range 0–20), with a mean number of 2.09 gains (range 0–12) and 2.06 losses (range 0–15). Frequent alterations (>10% of the cases) were gains on chromosome 2p16.3, 7p22.1-q36.3, 12p13.33-q24.33, 13q12.11-q34, 20p13-q13.33, and Xp11.21 and losses on chromosome 1p36-36.11, 17p13.1-p11.2, and 18p11.32-q23. Supplementary Table S1 shows an overview of common DNA copy number aberrations.

To discern any patterns of DNA copy number changes, unsupervised hierarchical cluster analysis was done, including all samples or only the 70 samples showing DNA copy number alterations. This revealed no significant association between cluster membership and phenotype (polyoid or flat, including different subtypes). Other clinicopathologic features, such as age, size, location, histology, and dysplasia grade, did not show associations with cluster membership.

Comparing flat versus polyoid adenomas using supervised univariate analysis revealed 6 regions to be statistically significantly different between the 2 phenotypes (FDR < 0.2). Losses of 1p36.32-p35.3, 10q25.3, 17p12, and 18p11.21-p23 were more frequent in polyoid adenomas than in flat adenomas, whereas losses at 5q14.3 and 5q15-q31.1 occurred more often in flat compared with polyoid adenomas (Table 2).

**APC mutation analysis**

The aCGH data revealed that flat adenomas more often showed a loss of chromosome 5q15-q31.1 than polyoid adenomas. This region harbors more than 100 genes and miRNAs (Supplementary Table S2), including APC which has an established role in CRC and was therefore investigated in more detail. Mutation analysis of the MCR was done for 35 polyoid adenomas (one with 5q loss, one with 5q gain, and 33 without 5q loss) and for 63 flat adenomas.
APC truncating mutations were present in 41.3% (26 of 63) of the polypoid adenomas, of which 2 adenomas contained a double truncating mutation, and in 62.9% (22 of 35) of the flat adenomas ($P = 0.04$; Supplementary Table S3 and Table 3).

Of the 13 flat adenomas with a 5q loss, 3 flat adenomas harbored an APC truncating mutation including one with a double mutation. The only polypoid adenoma that showed a gain on 5q harbored a truncating mutation. By comparing chromosomal alterations of all adenomas with and without a truncating mutation, loss of 5q15-q31.1 was the only significant region found (more present in adenomas without a truncating mutation, FDR < 0.2).

As mutations at different positions in APC resulted in different numbers of β-catenin downregulating motifs (20 amino acid repeats) in the remaining truncated protein, we also addressed this aspect in the current sample set. Six of the 26 mutated flat adenomas harbored one repeat and the other 20, two repeats. Of the 22 polypoid adenomas, 9 harbored one repeat and 13, two repeats. The number of repeats did not significantly differ between flat and polypoid adenomas ($P = 0.1$, Supplementary Table S3).

Table 2. Significantly different chromosomal regions (FDR < 0.2) between flat and polypoid adenomas

<table>
<thead>
<tr>
<th>Region</th>
<th>Location (bp)</th>
<th>Size (Mbp)</th>
<th>Gain/Loss</th>
<th>More loss/gain in Adenoma Type</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>1p36.32-p35.3</td>
<td>3520852–29506202</td>
<td>25.99</td>
<td>Loss</td>
<td>Polypoid</td>
<td>0.17</td>
</tr>
<tr>
<td>5q14.3</td>
<td>85676662–90745955</td>
<td>5.07</td>
<td>Loss</td>
<td>Flat</td>
<td>0.19</td>
</tr>
<tr>
<td>5q15-q31.1</td>
<td>100953123–133365906</td>
<td>32.41</td>
<td>Loss</td>
<td>Flat</td>
<td>0.17</td>
</tr>
<tr>
<td>10q25.3</td>
<td>115246724–117655181</td>
<td>2.41</td>
<td>Loss</td>
<td>Polypoid</td>
<td>0.17</td>
</tr>
<tr>
<td>17p12</td>
<td>15653683–15737241</td>
<td>0.08</td>
<td>Loss</td>
<td>Polypoid</td>
<td>0.19</td>
</tr>
<tr>
<td>18p11.21-q23</td>
<td>13925849–76116026</td>
<td>62.19</td>
<td>Loss</td>
<td>Polypoid</td>
<td>0.17</td>
</tr>
</tbody>
</table>

NOTE: Bold, regions altered in more than 10% of one of the two adenoma groups.
Gene ontology

Rather than focusing on one or a few genes at the significantly differently altered chromosomal regions, all genes located on these regions were entered into a gene ontology analysis. This provided insights in the genes and pathways potentially affected by these DNA copy number alterations (Table 2 and Supplementary Table S2). This resulted in a network top 5 that contained a significant overrepresentation of genes involved in genetic disorder, cell death, cellular development, hematologic system development, and function and hematopoiesis. When only the 5q15-q31.1 region was investigated, the network top 5 consisted of genetic disorder, inflammatory disease, gastrointestinal disease, cell death, and immunologic disease, thereby providing a possible association between the 5q15-q31 region and inflammation, which has also been described in inflammation-associated CRC (31).

Expression of CD3, CD8, and FoxP3

Previous studies in inflammation-associated CRC have shown more frequent 5q loss in these lesions in combination with less frequent APC mutations (32). To further explore this possible association of 5q loss/flat adenomas with inflammation, the amount of T-helper cells (CD3⁺CD8⁻), cytotoxic T cells (CD3⁺CD8⁺), and Tregs (FoxP3⁺) was investigated by triple fluorescent immunostaining in flat and polypoid adenomas (Fig. 2). Both lesion types showed TILs in the stroma; however in general, TILs were more abundant in the stroma of flat adenomas with chromosome 5q loss compared with polypoid adenomas (Fig. 2). Interestingly, when specifically looking at the amount of Tregs, an increase was noticed in flat adenomas with 5q loss compared with polypoid adenomas.

Serrated phenotype and MSI

Next to the chromosomal instability pathway, MSI is a second common pathway in CRC (12). Moreover MSI has been linked to serrated lesions (33), which often have a flat phenotype. In 114 of 118 adenomas, MSI status could be successfully determined. Of these, only one flat adenoma (0-IIa) showed MSI, excluding MSI as a major mechanism involved in the present cohort of flat adenomas. The present series contained in total 5 serrated flat adenomas (all MSS), whereas in the polypoid group no serrated adenomas were included. To exclude that the observed 5q loss was specific for serrated adenomas, these lesions were analyzed separately, which revealed that none of the serrated adenomas showed a 5q loss (Supplementary Fig. S2).

Other associations with DNA copy number changes

The different subgroups of flat adenomas were investigated in more detail. Of the 48 IIa, 3 IIc, 23 LST-F, and 9 LST-G cases, 8, 2, 3, and 1 sample(s), respectively, showed high-grade dysplasia (HGD) and 29, 0, 16, and 3 adenomas, respectively, showed more than 1% altered probes.

Table 3. Combined view of 5q loss and APC mutation data

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Polypoid adenomas (n = 35)</th>
<th>Flat adenomas (n = 83)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5q loss</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC mut</td>
<td>–</td>
<td>3 (1a)</td>
</tr>
<tr>
<td>APC WT</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>No data</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>5q normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC mut</td>
<td>21</td>
<td>23 (1a)</td>
</tr>
<tr>
<td>APC WT</td>
<td>12</td>
<td>29</td>
</tr>
<tr>
<td>No data</td>
<td>–</td>
<td>18</td>
</tr>
<tr>
<td>5q gain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APC mut</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>APC WT</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>No data</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: mut, APC truncating mutation; WT, APC without truncating mutation; –, no sample.

*Double mutation.

Figure 2. Triple fluorescent immunostaining of TILs.

A, polypoid adenoma without 5q loss. B, flat adenoma with 5q loss. The different TILs enumerated are T-helper cells (CD3⁺CD8⁻), cytotoxic T cells (CD3⁺CD8⁺), and Tregs (FoxP3⁺) (blue nucleus and red cell membrane).
In Supplementary Fig. S3, frequency plots of DNA copy number alterations are shown for the different subtypes. For 0-IIa and LST-F frequent DNA copy number alterations of both groups included gains on chromosomes 7, 13, and 20 and a loss on chromosome 5q. Between these 2 groups, no statistically significant difference was found, but when comparing these 2 subgroups to polypoid adenomas individually, losses at 5q14.3 and 5q15-q31.1 remained significantly different. Furthermore, between 0-IIa and polypoid adenomas, similar regions were found to be significantly different from those observed between flat and polypoid adenomas. Exceptions were the 1p region, which became more narrow (1p36.32-p35.3 toward 1p36.11), a new 0.08Mb deletion on chromosome 16 (16p31.3), and a larger region on chromosome 17 (17p12 toward 17p13.1-p11.2) harboring more genes, including TP53 (Supplementary Table S4).

In low-grade dysplasia (LGD) adenomas, only gains of chromosome 7 and 13 were common, whereas in HGD adenomas alterations on chromosomes 1, 4, 7, 8, 13, 17, 18, and 20 were frequently found (Supplementary Fig. S3), reaching statistical significance for the losses of 4q32.3-q35.2 and 17p13.1-p11.2 (Supplementary Table S4). As 12 of the 0-IIa adenomas originated from Japan, these samples were compared with the 36 IIA samples originating from Europe. Interestingly, gain of chromosome 7 was more frequent in European samples (Supplementary Fig. S3 and Supplementary Table S4). To exclude a possible bias in the comparison between flat and polypoid samples introduced by the different origin of the samples, the complete data analysis was repeated after excluding all Japanese samples. This, however, did not affect the significant differences found between flat and polypoid adenomas (data not shown).

Other clinicopathologic features, such as age, gender, histology, or location were not associated with any specific DNA copy number changes (Supplementary Fig. S2 and S3).

**Discussion**

Flat adenomas are associated with a more aggressive clinical behavior compared with their polypoid counterparts (6, 8). In particular, it has been suggested that these lesions would complete the adenoma-to-carcinoma progression at a higher speed (7). As differences in tumor phenotypes (based on the Paris classification), for a substantial part, are driven by their genotypes, this was sufficient reason for comparing a feature of tumor genotypes that is particularly associated with adenoma-to-carcinoma progression, that is, patterns of DNA copy number changes. The series of adenomas used for this study was collected in a multicenter setting, using advanced endoscopic techniques, and all lesions were annotated according to the Paris classification by trained gastroenterologists, who all had a high level of awareness of the existence of flat lesions. This study therefore represents the largest, representative, and well-characterized cohort of flat adenomas so far, in contrast to most studies in literature that frequently have small sample sizes and/or have favored selection of high-grade lesions.

DNA copy number changes were seen in around 60% of all flat as well as polypoid adenomas investigated in this study. This observation is in agreement with previous studies that also have shown that a part of adenomas did not show chromosomal aberrations (14, 34). To explore a possible confounding role of MSI in the present cohort, the MSI status was determined. In concordance with previous studies reporting MSI to be a rare event in (flat) adenomas, we observed only one flat adenoma with MSI in the present cohort (10, 35). The fact that MSI was not observed in serrated adenomas in this study is consistent with other studies and with the hypothesis that MSI is a late event in CRC tumorigenesis (36).

Next to CIN and MSI, a third epigenetic pathway has been described for CRC: CpG island methylator phenotype (CIMP; ref. 33). For the current sample cohort, the CIMP status was explored as well, showing that flat adenomas have a lower frequency of CIMP than polypoid adenomas (Voorham and colleagues; submitted for publication).

Many alterations observed in this study for flat and polypoid adenomas have been described before including gains on chromosome 7, 13, and 20, however not yet using a high resolution platform with multiple probes per gene (14, 34, 37–39). Supervised testing of chromosomal aberrations between flat and polypoid adenomas revealed that flat adenomas harbor a specific loss of 5q15.5-q31.1 and rarely show other losses that frequently occur in polypoid adenomas, that is, 1p36.32-p35.3, 10q25.3, 17p12, and chromosome 18.

In contrast to flat adenomas, loss of 1p and chromosome 18 are common alterations in CRC. Previously, loss of 1p has been described as an early and frequent event in CRC (40). Loss of 1p has also been linked to CRC tumor/stroma interaction, with a loss of 1p36 being associated with lower percentages of stroma in the tumor (41).

Loss of chromosome 18 has frequently been observed in CRC, and this chromosome harbors a number of important tumor suppressor genes, for example, DCC, SMAD4 (42, 43). In this study, chromosome 18 loss was less frequently found in flat adenomas and was associated with HGD, which is consistent with earlier findings of high prevalence of 18q in a series of flat adenomas enriched for high-grade lesions (38). Deletion of chromosome 18q has also been described as an important event in the transition from adenoma to carcinoma (42), which could indicate that flat adenomas acquire chromosome 18 loss later in their tumorigenesis. Consistent with these results, Nosho and colleagues (44) showed, by using expression arrays, significantly higher expression of SMAD4 in flat adenomas compared with polypoid adenomas.

The retention of the 5q15.5-q31.1 region in sporadic (polypoid) adenomas has been discussed before by Ried and colleagues (34). These authors proposed that possible mechanisms other than loss of 5q are important for the genesis of sporadic carcinomas. This study, however, indicates that for the carcinogenesis of flat lesions, the loss of 5q is relevant.
Although the 5q15.5-q31.1 region harbors many genes, APC is an obvious candidate to investigate in the context of CRC. APC is a key player in the Wnt signaling pathway, known to play a pivotal role in adenoma development. In this study, significantly more 5q loss and simultaneously significantly less APC-truncating mutations were observed in flat adenomas compared with polypoid adenomas. In line with this result, lower frequencies of APC mutations in depressed lesions have been described by Umetani and colleagues (9).

On the basis of the fact that flat lesions frequently have loss of the APC gene locus whereas polyoid lesions mostly have truncating mutations in APC, these 2 mechanisms of disruption of the APC gene seem to have a different biologic effect, which is reflected by the phenotype of the lesion.

It cannot be excluded that mutations outside the MCR of APC are present in flat adenomas. At the same time, other alternative mechanisms could affect the Wnt pathway as well, for example, methylation of APC or other genes and miRNAs involved in the Wnt pathway (45, 46). Also other candidate genes located on chromosome 5q15.5-q31.1 could be of interest. For example, MCC, which has been considered a candidate gene for FAP, before APC was discovered. This gene has recently been rediscovered as a putative tumor suppressor for serrated CRC and also has an effect on the Wnt signaling pathway (47). However, this is still in line with our notion that 5q deletion and APC mutation lead to a different phenotype.

Initially, all mutations in APC were considered to have equal impact on tumorigenesis. However, the "just-right" signaling model (48) shows that there is a strong selective pressure on the level of β-catenin signaling (due to different amounts of β-catenin degradation repeats remaining in the truncated protein) resulting from specific APC mutations. In this study, no significant difference in the type of mutations resulting in different amounts of remaining β-catenin degrading repeats was observed between flat and polypoid adenomas.

Loss of 5q has been described in colorectal carcinomas (49, 50), in which it has been associated with metastasis (42, 49, 50). The existence of 5q loss in flat lesions could explain the association with aggressiveness, which has been described for these lesions in the past (6, 8). On the other hand, it could also indicate that part of the colorectal carcinomas is originating from flat adenomas.

Loss of 5q and lack of APC mutation have been described before in ulcerative colitis, in which a 5q loss was found to be more prevalent in ulcerative colitis–associated CRC than in sporadic CRC (31, 51), and prevalence of APC mutations was found to be low in ulcerative colitis–associated CRC (32), as reported here for flat adenomas. In addition, based on gene ontology at the significantly different chromosomal regions between flat and polypoid adenomas, genes involved in inflammation were significantly overrepresented. Therefore, we speculate that there are parallels between the molecular biology of flat adenomas and ulcerative colitis–associated CRC. Of note, patients with a history of IBD were excluded from this study, precluding this source of bias. Interestingly, the lost 5q region in flat adenomas was partly overlapping with the IBD5 locus (5q31-33), which was suggested to be one of the most important genetic factors involved in the pathogenesis of IBDs (52). This locus harbors a cytokine gene cluster that includes interleukins (IL) 3, 4, 5, and 13 and colony-stimulating factor-2 (CSF-2). Because both in inflammation-associated CRC and flat adenomas this locus is lost, lower expression of these ILs may be important for the development of both types of colorectal neoplasia. Consistent with this hypothesis, we found an increase of infiltrating T cells, in particular immunesuppressing Tregs in the stroma of flat adenomas with a 5q loss. As Tregs are involved in immune evasion, this feature could also affect tumor aggressiveness (53). Further validation of these findings is necessary to determine whether increased presence of Tregs is characteristic for flat lesions in general. Furthermore, experimental evidence for the link between flat lesions and colitis-associated colorectal neoplasia comes from the dextran sulphate sodium colitis mouse model in which flat lesions were associated with more severe inflammation than polyoid lesions by several groups (54–56). A potential factor could be COX-2, which is proinflammatory and has been shown to be higher expressed in flat lesions compared with polyoid lesions (57). These findings support the hypothesis that, especially in relation to 5q loss, flat colorectal adenomas are more similar to colitis-associated colorectal neoplasia, than to polyoid adenomas.

Despite the relatively large amount of flat adenomas in this study, the number of lesions from specific subtypes (i.e., IIc, LST-G, and LST-F) was low, which may be because of the fact that this was a consecutive series and that overall these subtypes are less common. In this exploratory study, the number of IIc and LST-G adenomas was too low to draw meaningful conclusions. However, both in LST-F and 0-IIa, still 5q loss was significantly more common than in polyoid adenomas.

The high resolution array platform used in this study has excellent performance for detecting DNA copy number changes when using FFPE material (58), in contrast to the SNP array platforms available at the time of the study. However, this platform cannot detect copy-neutral LOH, an event frequently reported in CRC (59), and therefore this could not be investigated. Moreover, because of the lack of matched normal DNA and the lack of enough DNA, determining LOH using PCR-based techniques was not feasible.

In summary, polypoid and flat adenomas show partly distinct chromosomal profiles, supporting a different molecular biology. In particular, flat lesions frequently displayed loss of chromosome 5q, which has previously been associated with an aggressive clinical course, consistent with the supposed more aggressive behavior of flat lesions. On the basis of our findings, we postulate that parallel pathways might be involved in the biology of flat colorectal neoplasia and IBD-associated CRC. This study warrants further investigations into the possible involvement of inflammation in the molecular biology underlying flat lesions.
Disclosure of Potential Conflicts of Interest

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References


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