Chromosome 5q loss in colorectal flat adenomas

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**Abbreviations:** aCGH, array comparative genomic hybridization; APC, adenomatous polyposis coli; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CNV, copy number variation; CRC, colorectal cancer; FAP, familial adenomatous polyposis; FDR, false discovery rate; FFPE, Formaldeyde-fixed, paraffin-embedded; HGD, high grade dysplasia; IBD, inflammatory bowel disease; LGD, low grade dysplasia; LOH, loss of heterozygosity; LST-F, lateral spreading type flat; LST-G, lateral spreading type granular; MSI, microsatellite instability; TILs, tumor infiltrating lymphocytes; Tregs, regulatory T-cells; WECCA, Weighted Clustering of Called aCGH data.
TRANSLATION RELEVANCE

Flat lesions are a distinct type of colorectal lesions representing a substantial part of all CRC lesions. They are associated with a more aggressive clinical behavior compared to 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 to polypoid adenomas and at the same time they show significantly less APC truncation mutations. These two 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.
ABSTRACT

Purpose
Flat adenomas are a subgroup of colorectal adenomas that have been associated with a more aggressive clinical behavior compared to their polypoid counterparts. Here, we aimed to compare one of the molecular changes most explicitly associated with adenoma to carcinoma progression, i.e. 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 arrayCGH platform, microsatellite instability (MSI) status and for mutations in the adenomatous polyposis coli (APC) gene. Immunohistochemical stainings for CD3, CD8 and FoxP3 expression were performed.

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, while losses of 1p36.32-p35.3, 10q25.3, 17p12 and chromosome 18 were more frequent in polypoid adenomas (FDR<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 to 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.
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 et al demonstrated 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 non-polypoid precursors of sporadic colorectal cancer 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%-36%(4-6). For the GI community at large this has changed only recently with publications on large US based series of flat adenomas(6).

For obvious reasons, flat adenomas can more easily go undetected during colonoscopy, especially when bowel preparation is sub-optimal 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(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 figure 1(6,8). Formal estimations of progression risk would require unethical longitudinal studies leaving adenomas untreated. However, it is feasible to determine the biological 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 methodological 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 pathway (MSI), where 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 two adenoma phenotypes. Therefore, the aim of the present study was to compare DNA copy number aberrations between flat and polypoid adenomas in a well-defined series using a high resolution 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 five different institutes. Exclusion criteria were patients with hereditary forms of CRC (including familial adenomatous polyposis (FAP) and hyperplastic polyposis syndrome (HPS)), inflammatory bowel disease (IBD), hyperplastic polyps, adenoma size smaller than 5 mm (except for two depressed adenomas, subtype 0-IIc, since these were of clinical interest) and insufficient DNA quality, resulting in a series of 83 flat adenomas and 35 polypoid adenomas. All lesions were detected by selective chromoendoscopy (dye-spraying) which has been suggested to enhance the detection rate of flat lesions(15). Of these 118 cases, 37 adenomas from 32 patients were from Leeds General Infirmary, Leeds, UK, collected between 1995-2007(5), 17 adenomas, from 16 patients were from Hospital Vitkovice, Ostrava, Czech Republic, collected between 2006-2008(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 2006-2008. 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) the present 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), where LST is a subclass of the IIa 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. Histological information was obtained by revising all HE-slides by one pathologist according to European guidelines(18) to avoid potential inter-observer bias (Table1). Major histological 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 figure 1).

**DNA isolation**

DNA from FFPE material was isolated following macro-dissection (> 70% dysplastic cells) as described before(21) with a few modifications. A five day incubation period with lysis buffer (ATL buffer, QI Amp, DNA micro-kit, Qiagen, Venlo, The Netherlands) and freshly added (once every day) proteinase K (10 µl of 20 ng/µl) was performed. DNA was isolated the QI Amp DNA micro-kit (Qiagen) and concentrations and purity was measured on a Nanodrop ND-1000 spectrophotometer (Isogen, IJsselstein, The Netherlands).

**DNA quality control and aCGH**

DNA quality was tested using the CGH Labeling Kit for Oligo Arrays (Enzo Life Science, Farmingdale, NY) where a specific activity (pmol per µl dye/µg per µl genomic DNA) lower than 30 pmol/µg was considered insufficient and excluded for aCGH. Hybridizations were performed using arrays that contained 180880 in situ synthesized 60-mer oligonucleotides (4x180K, GPL8687, Agilent Technologies, Palo Alto, CA), representing 169793 unique chromosomal locations evenly distributed across the genome (space ~ 17kb) and 4548 additional unique oligonucleotides, located at 238 of the Cancer Census genes.
Labeling, hybridization, scanning and feature extraction were performed as previously described for the 4x44K 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 performed according to the across aCGH approach as described before(22). As reference samples normal DNA from resection margins of 11 male or 11 female non-cancer patients were used. Data is made publicly available in GEO (www.ncbi.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 CGHCcall (version 2.5) with SD-Undo of 2 and SD-Undolong of 3. Segments with a probability score of > 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 (CNVs). Since 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, 23268 probes were removed, leaving 151073 probes to be analyzed. Of the removed probes, 98.5% was present in the Database of Genomic Variants (http://projects.tcag.ca/variation/)(25,26).

CGHRegions(27) was used to reduce the dimension of the dataset, accepting maximally 0.1% information loss (threshold=0.001). The use of regions improves the effectiveness of the subsequent statistical analyses and facilitates the interpretation of the results.

**Microsatellite instability status**

MSI analysis was performed using the MSI Analysis System (MSI Multiplex System Version 1.2, Promega, Madison, WI, USA) consisting of five quasi-monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24, MONO-27) according to manufacturer’s instructions. PCR products were separated using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), 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 two or more markers were instable, the sample was interpreted as microsatellite instable (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 four flanking PCRs followed by two semi-nested PCRs, as described(29).
Sequencing was done on a 3500 Genetic Analyzer (Applied Biosystems Foster city, CA, USA) 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 (TILs)**

Simultaneous immunohistochemical staining of three different epitopes was applied to 4-µm FFPE sections of 9 polypoid 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 546, 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 TILs. 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 performed(27).

A binomial test on differential proportions of alterations was performed (CGHTest; www.few.vu.nl/~mavdwiel). This test procedure includes a permutation-based false discovery rate (FDR) correction for multiple testing. Alterations occurring 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 polypoid lesions were evaluated by Chi-square test.
Gene ontology analysis

To interpret the biological relevance of the genes located at the altered chromosomal regions, a gene ontology analysis was performed (Ingenuity Systems, Redwood City, USA). Biological/molecular functions were considered to be significantly overrepresented when they contained more than 20 genes and the Benjamini-Hochberg corrected p-value was p<0.1.
RESULTS

DNA copy number changes

In the present study we compared high-resolution genome-wide chromosomal profiles of a well-defined series of flat and polypoid colorectal adenomas. Overall the adenoma-size was similar between the two groups (p=0.4). To determine common chromosomal alterations, the frequencies of gains and losses per probe were plotted for flat and polypoid adenomas separately (Figure 1). Overall both phenotypes showed low frequencies of DNA copy number aberrations. Forty-eight of the 83 flat adenomas (58%) and 22 of 35 polypoid adenomas (63%) had DNA copy number aberrations for more than 1% of 151073 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. Polypoid 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, 18p11.32-q23. Supplementary table 1 shows an overview of common DNA copy number aberrations.

To discern any patterns of DNA copy number changes, unsupervised hierarchical cluster analysis was performed including all samples or only the 70 samples showing DNA copy number alterations. This revealed no significant association between cluster membership and phenotype (polypoid or flat, including different subtypes). Other clinicopathological features, such as age, size, location, histology and dysplasia grade, did not show associations with cluster membership.
Comparing flat versus polypoid adenomas using supervised univariate analysis, revealed 6 regions to be statistically significantly different between the two phenotypes (FDR<0.2). Losses of 1p36.32-p35.3, 10q25.3, 17p12 and 18p11.21-p23 were more frequent in polypoid adenomas than in flat adenomas, while losses at 5q14.3 and 5q15-q31.1 occurred more often in flat compared to polypoid adenomas (Table 2).

**APC mutation analysis**

The aCGH data revealed that flat adenomas more often showed a loss of chromosome 5q15-q31.1 than polypoid adenomas. This region harbors over 100 genes and microRNAs (supplementary table 2), including *APC* which has an established role in CRC and was therefore investigated in more detail. Mutation analysis of the MCR was performed for 35 polypoid adenomas (one with 5q loss, one with 5q gain, 33 without 5q loss) and for 63 flat adenomas (13 with 5q loss, 50 without 5q loss). *APC* truncating mutations were present in 41.3% (26/63) of the polypoid adenomas, of which two adenomas contained a double truncating mutation, and in 62.9% (22/35) of the flat adenomas (p=0.04)(Supplementary table 3 and table 3).

Of the 13 flat adenomas with a 5q loss, three flat adenomas harbored an *APC* truncating mutation including one with a double mutation. The only polypoid adenoma which 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* result in different numbers of β-catenin down-regulating 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 3).

**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 2). This resulted in a network top 5 that contained a significant overrepresentation of genes involved in genetic disorder, cell death, cellular development, hematological 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 immunological 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 demonstrated 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 regulatory T-cells (Tregs, FoxP3⁺) was investigated by triple fluorescent immunostaining in flat and polypoid adenomas (Figure 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 to polypoid adenomas (Figure 2). Interestingly, when specifically looking at the amount of Tregs an increase was noticed in flat adenomas with 5q loss compared to 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 out 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 five 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 figure 2).

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.

In supplementary figure 3, 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 two groups, no statistically significant difference was found, but when comparing these two subgroups to polypoid adenomas individually, losses at 5q14.3 and 5q15-q31.1 remained significantly different. Furthermore, for 0-IIa, similar regions were found to be significantly as were 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 towards 17p13.1-p11.2) harboring more genes, including TP53 (Supplementary table 4).

In low grade dysplasia (LGD) adenomas, only gains of chromosome 7 and 13 were common, while in HGD adenomas alterations on chromosomes 1, 4, 7, 8, 13, 17, 18, 20 were frequently found (supplementary figure 3), reaching statistical significance for the losses of 4q32.3-q35.2 and 17p13.1-p11.2 (Supplementary table 4).

As 12 of the 0-IIa adenomas originated from Japan, these samples were compared to the 36 IIa samples originating from Europe. Interestingly, gain of chromosome 7 was more frequent in European samples (Supplementary figure 3 and Supplementary table 4). 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 clinicopathological features, such as age, gender, histology or location were not associated with any specific DNA copy number changes (supplementary figure 2 and 3).
DISCUSSION

Flat adenomas are associated with a more aggressive clinical behavior compared to 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, i.e. patterns of DNA copy number changes. The series of adenomas used for the current 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. The present 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 the present 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 the current 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)(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 et al., submitted for publication).

Many alterations observed in the present 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 i.e. 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, e.g. DCC, SMAD4(42,43). In the current 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 to polypoid adenomas.

The retention of the 5q15.5-q31.1 region, in sporadic (polypoid) adenomas has been discussed before by Ried et al.(34). These authors proposed that possible mechanisms other than loss of
5q are important for the genesis of sporadic carcinomas. The current study, however, indicates that for the carcinogenesis of flat lesions, the loss of 5q is relevant.

While 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 the present study significantly more 5q loss and simultaneously significantly less APC truncating mutations were observed in flat adenomas compared to polypoid adenomas. In line with this result, lower frequencies of APC mutations in depressed lesions have been described by Umetani et al(9).

Based on the fact that flat lesions frequently have loss of the APC gene locus, whereas polypoid lesions mostly have truncating mutations in APC, these two mechanisms of disruption of the APC gene seem to have a different biological 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, e.g. methylation of APC or other genes and microRNAs 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 colorectal cancer 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 the current
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), where 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 (UC), where a 5q loss was found to be more prevalent in UC-associated CRC than in sporadic CRC(31,51) and prevalence of APC mutations was found to be low in UC-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 UC-associated CRC.

Of note, patients with a history of IBD were excluded from the present 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 inflammatory bowel diseases(52). This locus harbors a cytokine gene cluster that includes interleukins 3,4,5 and 13 and colony-stimulating factor-2 (CSF-2). Since both in inflammation-associated CRC and flat adenomas this locus is lost, lower expression of these interleukins 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 immune-suppressing 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 (DSS) colitis mouse model where flat lesions were associated with more severe inflammation than polypoid lesions by several groups(54-56). A potential factor could be COX-2, which is pro-inflammatory and has been shown to be higher expressed in flat lesions compared to polypoid 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 polypoid adenomas.

Despite the relatively large amount of flat adenomas in the current study, the number of lesions from specific subtypes (i.e. IIc, LST-G and LST-F) was low, which may be due to 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 polypoid adenomas.

The high resolution array platform used in the present 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, due to 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. Based on our findings we postulate that parallel pathways might be involved in the biology of flat colorectal neoplasia and inflammatory bowel disease-associated colorectal cancer. This study warrants further investigations into the possible involvement of inflammation in the molecular biology underlying flat lesions.
Acknowledgment:
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Combined array-comparative genomic hybridization and single-nucleotide
Table 1; Clinical characteristics of the 118 adenomas used for aCGH analysis. Patients with familial forms of CRC, inflammatory bowel disease (IBD), or hyperplastic polyps were excluded. LGD, low grade dysplasia; HGD, high grade dysplasia.
<table>
<thead>
<tr>
<th>Region</th>
<th>Location (bp)</th>
<th>Size (Mbp)</th>
<th>Gain/Loss</th>
<th>More loss/gain in</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>85676562-90745955</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>

Table 2; Significantly different chromosomal regions (FDR<0.2) between flat and polypoid adenomas; FDR, False discovery rate; Bold, regions present in more than 10% in one of the lesion types.
<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 (1*)</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 (1*)</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>

Table 3; Combined view of 5q loss and *APC* mutation data. *, double mutation; mut, *APC* truncating mutation; WT, *APC* without truncating mutation; -, no sample.
FIGURES LEGENDS

Figure 1; Frequency plot of flat and polypoid adenomas. The frequencies of gains (positive Y-axis) and losses (negative Y-axis) for chromosome 1-23 determined by aCGH shown for each probe for both adenoma groups (top, 35 polypoid adenomas; bottom, 83 flat adenomas). Significant differential chromosomal regions (FDR<0.2) between the groups are indicated by black rectangles when more present in the polypoid adenomas and grey rectangles when more present in flat adenomas.

Figure 2; Triple fluorescent immunostaining of tumor infiltrating lymphocytes (TILs). A, polypoid adenoma without 5q loss. B, flat adenoma with 5q loss. The different TILs enumerated are T-helper cells (CD3+CD8- cells, red), cytotoxic T-cells (CD3+CD8+, yellow) regulator T-cells (Tregs, FoxP3+ cells (blue nucleus and red cell membrane))
Chromosome 5q loss in colorectal flat adenomas

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