Detection and Characterization of Flat Aberrant Crypt Foci (Flat ACF) in the Novel A/J Min/+ Mouse

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Abstract. *Background/Aim: Flat aberrant crypt foci (flat ACF) and mucin-depleted foci (MDF) have previously been described as preneoplastic colonic lesions. We used the novel A/J Min/+ mouse model, that demonstrates extensive spontaneous colon carcinogenesis to refine the method of detection of flat ACF and further characterize and define them as early lesions by histological examination and comparison with MDF. Materials and Methods: Colons were stained with methylene blue (MB) for flat ACF detection and restained with high-iron diamine-alcian blue (HID-AB) for MDF detection. Results: Optimal flat ACF recognition required at least 24 h of storage post-MB staining and adherence to a set of characteristics. The fraction of flat ACF corresponding with MDF was 93%. Flat ACF/MDF displayed the same picture of severe dysplasia, lack of mucus and goblet cells and accumulation of cytoplasmic β-catenin. Conclusion: The easily detectable flat ACF are reliable surface biomarkers of Apc-driven colon carcinogenesis.*

To date, a variety of preneoplastic lesions thought to be the initial step in colon carcinogenesis have been characterized, including aberrant crypt foci (ACF), flat aberrant crypt foci (flat ACF) and mucin-depleted foci (MDF) (1-4).

Aberrant crypt foci (ACF) were originally described by Bird in 1987 in the colon of mice treated with carcinogens and are identified by their characteristic morphology of enlarged crypts, thickened epithelial lining, irregular lumens, increased pericryptal space and elevation from the mucosa (1, 5). MDF were first described by Caderni *et al*. in 2003 (4) in the colon of azoxymethane (AOM)-treated rats and found to show dysplastic properties similar to those observed in colonic tumors. This lesion is first and foremost characterized by no- or scarce production of mucins. Other features of this lesion include distorted cryptal lumens that are smaller than surrounding crypts, elevation from the mucosa and crypt multiplicity of more than 3 crypts (4, 6). Furthermore, MDF also show both β-catenin accumulation and *Apc* mutations, two traits present in colonic tumors, further emphasizing MDF as preneoplastic lesions for colon carcinogenesis (7, 8).

The multiple intestinal neoplasia (Min/+) mouse is, like humans with the familial adenomatous polyposis (FAP) syndrome, heterozygous for a mutation in the tumor suppressor gene *Apc*. This mutation leads to the development of numerous neoplastic intestinal polyps. Inactivation of *Apc* with β-catenin accumulation mimics Wnt signaling pathway stimulation (10-12). Unlike human FAP, conventional C57BL/6J (B6) Min/+ mice develop tumors predominantly in the small intestine. Even though the incidence of colonic tumors is very low in this mouse, preneoplastic colonic lesions, called flat ACF, were found in the colons of young B6 Min/+ mice; however, no ACF were detected (3). Flat ACF differed from the 'classic' ACF described by Bird as they were not elevated from the mucosa and they were undetectable without methylene blue (MB) staining and transillumination. Later studies in azoxymethane (AOM)-treated B6 Min/+ mice and rats revealed that only the flat ACF, not the classic ACF, demonstrated a continuous development from monocryptal stage to tumor (2, 12). In AOM-treated B6 Min/+ mice scored for flat ACF, MDF and classic ACF, a 57% correspondence between flat ACF and MDF was observed. Approximately, 50% of the non-coincident lesions were identified as classic ACF, while the rest were not (13).

Our novel Min/+ mouse on an A/J genetic background provides a better model for colorectal cancer as these mice spontaneously develop a considerable number of colonic lesions that demonstrate a continuous growth from the monocryptal stage to adenoma and, with time, carcinoma (14). Flat ACF are seldom chosen when colonic lesions are to be scored, mainly due to a notion that this particular lesion is difficult to detect (15).

The objectives of the present study were to: (i) refine the method of detection for flat ACF using the sensitive A/J Min/+ mouse; (ii) further characterize the flat ACF and define them

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as early lesions of colon carcinogenesis by histological examination and comparison with MDF.

Materials and Methods

A/J Min/+ mice. Intestines collected from A/J Min/+ mice bred at the Department of Experimental Biomedicine at the Norwegian University of Life Sciences, Campus Adamstuen, were used for the study. All animals were housed in Makrolon Type III open top plastic cages in a room with a 12-h light/dark cycle, 55-65% humidity and 20-22˚C. Water and feed were given *ad libitum*. All animals were fed the standard rodent diet RM1 (SDS Special Diet Services, Witham, UK) for the duration of the study. Mice were sacrificed by cervical dislocation. This study was conducted in strict accordance with The Norwegian Regulation on Animal Experimentation and approved by the Institutional Animal Care and Use Committee at the Norwegian University of Life Sciences, Campus Adamstuen.

Methylene blue (MB) staining and scoring of flat ACF. The colon was excised from anus to cecum, rinsed in and flushed with ice-cold phosphate buffered saline solution (PBS) to remove any intestinal contents before being slit open longitudinally. Next, the colon was fixed flat between two PBS-soaked filter papers held together with staples. The flat-fixed colon was then stored in 10% neutral buffered formalin for at least 24 h before staining. Once fixated, the colon was removed from the filter paper. Any remaining fat on the muscularis side of the colon was carefully removed with a pair of forceps before the colon was stained for 8-10 sec in a glass beaker containing a 0.2% MB (M9140; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 10% neutral buffered formalin solution. After no more than 10 sec, the colon was transferred to a new beaker filled with 10% formalin, rinsed of excess MB before again being transferred to another beaker containing 10% formalin for a second rinse. Next, the colon was moved to an individual 50-ml Falcon® polypropylene tube filled with either 10% formalin or 70% ethanol for at least 24 h. This last step is of importance because the flat ACF are barely visible until after at least 24 hours post-MB staining (Figure 1a). To examine the MB-stained intestine, the colon was placed in a Nunc® OmniTray single-well plate (Thermo Fisher Scientific, Waltham, MA, USA). This plate was divided into segments by carving vertical lines, each 1 cm thick, down the length of the plate. Additionally, one horizontal line spanning the plate was drawn down the center of the plate, thus dividing the plate into a top and bottom section. This grid was used to map the location of all colonic lesions. The colon, with the mucosal side down, was fixed under a glass microscope slide to ease scoring. To examine the colon for flat ACF, surface microscopy and transillumination using an inverted microscope (CKX41; Olympus Inc., Hamburg, Germany) equipped with a digital color camera (DP25; Olympus Inc.) was used. Morphological appearance and surface location of each lesion were documented with an image and grid coordinates.

To be considered as flat ACF, a lesion must fulfill certain specific criteria. It must (i) turn a bright blue/green color after MB staining, a coloration distinguishable from normal crypts, which stain a more subdued brownish-green, (ii) have enlarged crypts with compressed luminal openings, (iii) show elongated pit patterns, which gives it a gyrus-like appearance. One additional feature of flat ACF is that the majority of the lesions lay flat against the surrounding epithelium in the colon; however, a small number of lesions may appear somewhat polypoid. To be considered as flat ACF, the first three criteria must be met, while flatness, albeit the lesion's name suggesting so, is not required. Although flat ACF can be detected from the monocryptal stage, in order to compare this lesion to the MDF, only flat ACF containing more than 3 crypts were scored for the comparison analysis; thus, all lesions between 4 and 30 crypts were scored.

High-iron diamine-alcian blue (HID-AB) staining and scoring of MDF. After scoring the MB-stained colon for flat ACF, the same colon was stained with the HID-AB mucin stain to look for MDF. HID stains sulfomucins dark brown, while AB stains sialomucins blue (16). Other than the amount of diamines used, the staining procedure was as described by Caderni *et al*. (17). Briefly, the MB-stained colons were rinsed in distilled water for 5 minutes before staining with a HID solution made with 45 mg N-N'-dimethyl-m-phenylenediamine (219223; Sigma-Aldrich) and 55 mg of N-N'-dimethyl-p-phenylenediamine (D4139; Sigma-Aldrich) dissolved in 50 ml of distilled water, with 1.4 ml of 60% ferric chloride. Each colon was stained for no less than 18 hours at room temperature, rinsed three times in distilled water and stained for 30 min with 1% AB (A1357; Sigma-Aldrich) in 3% acetic acid. Next, colons were rinsed in 80% ethanol three times followed by a rinse in distilled water and stored in 10% formalin or 70% ethanol. HID-AB-stained colons were examined like the MB-stained intestines and morphological appearance, as well as surface location of each MDF were documented with an image and grid coordinates, which were later compared to the corresponding information gathered from the MB-stained colon. All MDF larger than 3 crypts, and smaller than 30 crypts, were scored for comparison with flat ACF.

Histological examination. After scoring the MB- and HID-AB-stained intestines, histological cross-sections were prepared. The HID-AB-stained intestines were cut into 1 cm segments from distal to proximal end and embedded in paraffin with the muscle side down before histological cross sections (2-3 μm) cut in parallel with the mucosal surface were made. The sections were stained with hematoxylin and eosin (HE). A set of intestines only stained with MB were also used. These intestines were prepared with a modification of the Swiss roll technique (18). Briefly, the longitudinally cut, flat-fixed colon was rolled lengthwise, from proximal to distal end with the mucosa facing inward, using a pair of curved dissecting forceps. To hold the roll together, a pin was pushed carefully through it. The prepared Swiss rolls were embedded in paraffin before histological sections (2-3 μm) were made. Each section was then manually stained with both MB and HID-AB. The sections were stained with MB and HID-AB to observe how histological sections of flat ACF and MDF stain as compared to whole colon staining. For MB staining: the histological sections were stained for 10 seconds in the same MB solution used to stain the full colon, followed by two 10% formalin rinses before overnight storage in 10% formalin. For HID-AB staining: the sections were stained in the equivalent HID solution as used for the colon whole mount for no less than 18 hours and, subsequently, rinsed in dH_2O prior to a dip in AB. Next, the sections were rinsed in dH2O again and counterstained for 5 minutes with nuclear fast red to achieve the pink coloration seen in the whole mount sample scored for MDF. Finally, before xylene mounting, the sections were rinsed in dH_2O.

Immunohistochemistry. Swiss rolls were also subjected to β-catenin staining to show Wnt signaling activation. Paraffin sections were deparaffinized and rehydrated before endogenous peroxidase quenching with 3.0% H_2O_2. Antigen retrieval was performed in Tris/EDTA

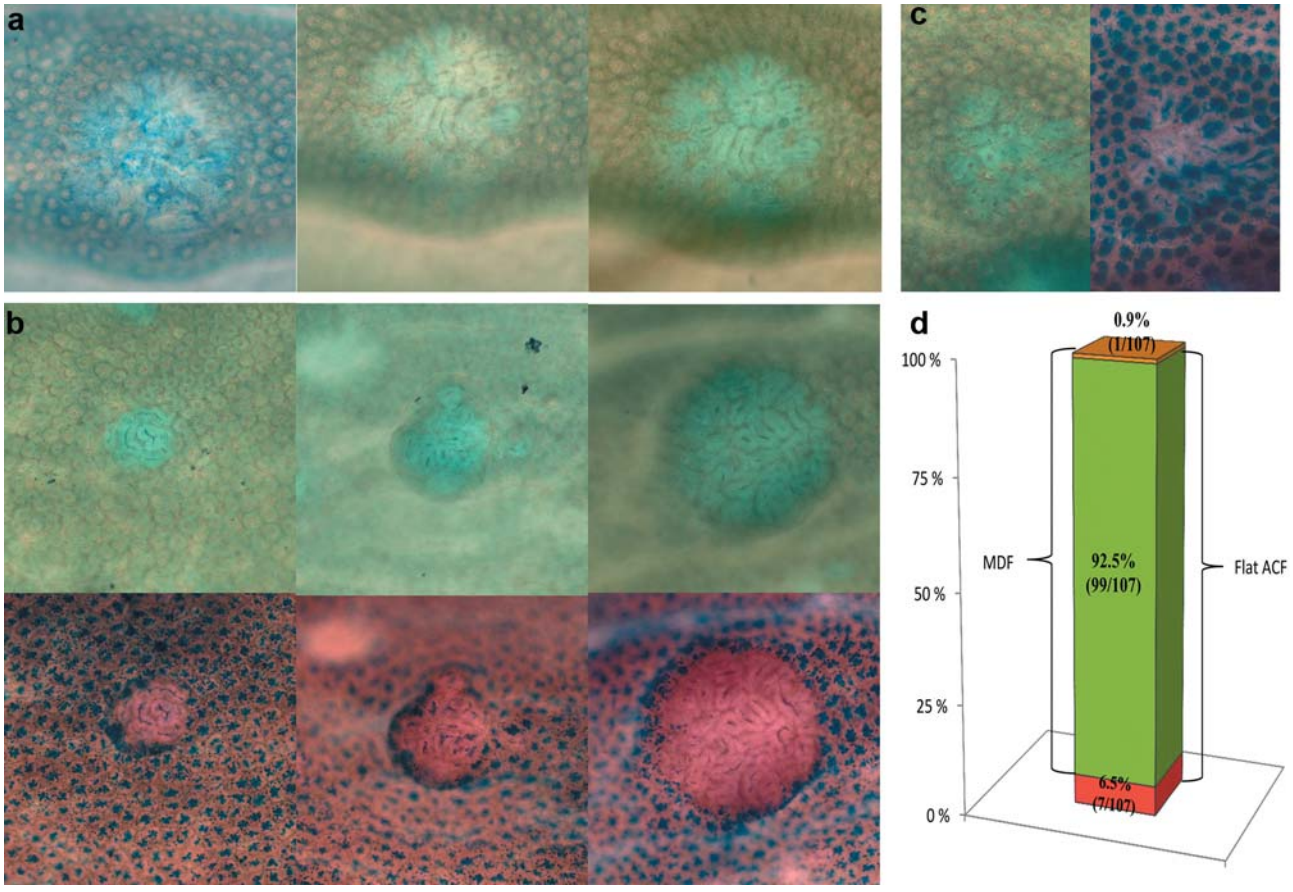

Figure 1. *Representative examples of morphological features of colonic lesions spontaneously formed in the A/J Min/+ mouse. Images (a; far right) and (b; top row) are good examples of flat ACF fulfilling the criteria of coloration, crypt size, compressed luminal openings and gyrus-like pit patterns. (a) Illustrates the importance of allowing sufficient time to pass after staining the colon with methylene blue (MB) prior to scoring: coloration of a large flat ACF lesion after 10 min (left), 3 h (middle) and 24 h (right). (b) Coinciding lesions identified as flat ACF with MB staining (top) and as MDF with high-iron diamine-alcian blue (HID-AB) staining (bottom). The middle image shows a polypoid flat ACF/MDF lesion that is slightly elevated from the mucosa. (c) A non-corresponding lesion scored as flat ACF (left) but not as MDF (right). (d) Histogram showing the percent of flat ACF scored in MB-stained colons corresponding with MDF scored in HID-AB-stained colons (green box), percent of flat ACF not scored as MDF in HID-AB (red box) and percent of MDF not corresponding with flat ACF in MB (orange box). Values in parentheses show the number of lesions in each category. All magnifications are ×100.*

(pH 9.1). Sections were blocked using the Mouse on Mouse (M.O.M) kit (Vector Laboratories, Burlingame, CA, USA) before incubation with a primary monoclonal antibody against β-catenin (Purified mouse anti-β-catenin, C19220; Transduction Laboratories, Lexington, KY, USA) at a 1:2,500 dilution. Sections were incubated in M.O.M. biotinylated anti-mouse IgG reagent followed by addition of the avidin–biotinylated peroxidase complex. Antibody binding was detected with DAB substrate according to the manufacturer's protocol (34065; Thermo Scientific Pierce, Waltham, MA, USA). Sections were counterstained with hematoxylin, and mounted from xylene.

Results

Detection of flat ACF. Flat ACF were easily identified when the colons were prepared as described in detail above. Critical steps for detection were: flushing through the colon with ice cold PBS to remove luminal content; longitudinal bisection of the colon before careful flattening on a smooth, PBS-soaked surface; transferring the wet, flat colon preparation to filter paper and placing another wet filter paper on top, securing with staples; fixing flat between the filter papers in formalin for at least 24 h. Testing showed that staining with MB for no more that 8-10 sec, followed by at least 24 h of storage before surface examination, produced optimal lesion coloration. When adhering to these time requirements, flat ACF appeared as blue-green structures that differed from the surrounding tissues, which stained a brownish-green color (Figure 1a). In addition to coloration, compressed crypt openings exhibiting gyrus-like pit patterns (Figure 1a and b) were considered critical features for flat ACF detection. Furthermore, the majority of flat ACF

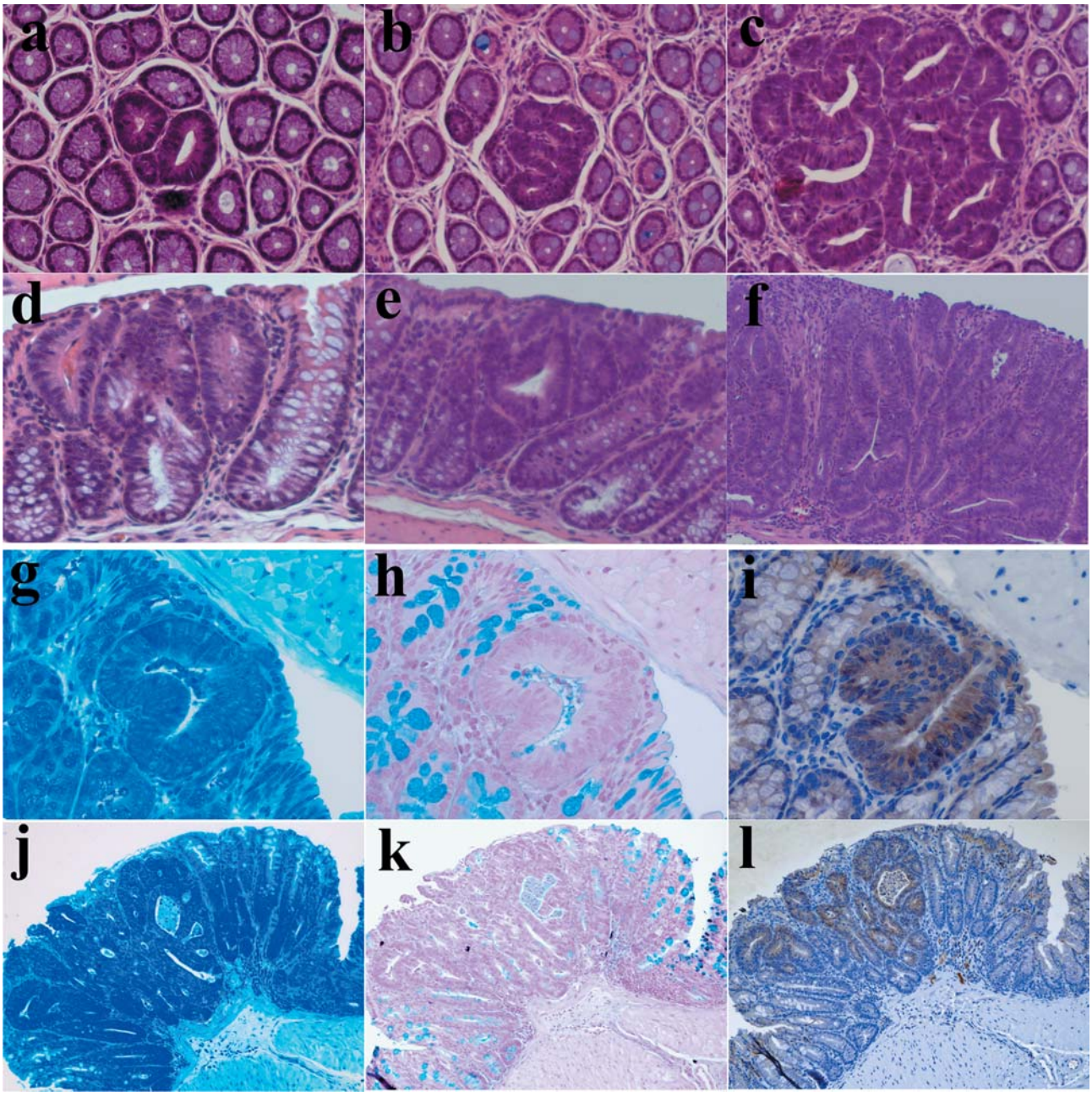

Figure 2. *Representative examples of histological features of colonic lesions spontaneously formed in the A/J Min/+ mouse. (a-c) Cross-sections of HE-stained flat ACF of different sizes. Longitudinal sections of HE-stained Swiss rolls showing small flat ACF (d and e) and a tumor (f); note that both small and large lesions (a-e, f) display the same degree of dysplasia. Longitudinal sections of Swiss rolls stained with (g and j) methylene blue (MB), (h and k) high-iron diamine-alcian blue (HID-AB) counterstained with nuclear fast red and (i and l) ß-catenin immunohistochemistry. (g-i) shows a 1-2 crypt flat ACF/MDF, while (j-l) shows a large flat ACF/MDF lesion. HID-AB staining (h and k) clearly allowed for detection of the dysplastic lesions by indicating loss of goblet cells and mucus production, while MB staining (g and j) merely stained the dysplastic crypts a darker blue than normal crypts. β-catenin accumulation (i and l) was observed in sections coinciding with the MB and HID-AB lesions, indicating that both show Wnt signaling pathway activation. Magnifications (a-e) and (g-i): ×400; Magnifications (f) and (j-l): ×100.*

exhibited a flat structure compared with the surrounding mucosa, even at a relatively large size (Figure 1b, right); however, sometimes these lesions could have a polypoid appearance, even when small in size (Figure 1b, middle). Polypoid flat ACF were differentiated from classic ACF by their gyrus-like pit pattern.

Correspondence between flat ACF and MDF. Ten colons from 10- to 32-week-old A/J Min/+ mice were examined after staining with two distinct staining methods to detect the early colonic lesions, flat ACF and MDF. A total of 107 lesions were scored. Of these, 106 were scored as flat ACF and 100 were scored as MDF: 99 lesions were scored as flat ACF in MB and as MDF in HID-AB, with a fraction of correspondence of 92.5% (Figure 1b and d). Seven lesions (6.5%) were scored as flat ACF in the MB-stained intestine, but did not fulfill the criteria of MDF after HID-AB staining. Also, 0.9% of lesions that were identified as MDF in the HID-AB-stained colon were not scored as flat ACF in the MB-stained intestine (Figure 1d). The non-corresponding lesions were either not detected or did not fulfill criteria for the specified lesion (Figure 1c).

Histological characterization of corresponding flat ACF and MDF. After surface examination, histological cross-sections were performed to determine the pathological status of the lesions. All the flat ACF/MDF examined exhibited the same picture of severe dysplasia as shown in Figure 2a-c. Longitudinal sections made from Swiss rolls demonstrated that small and large lesions always displayed the same degree of dysplasia (Figure 2d-f). Serial sections from Swiss rolls were stained with MB, HID-AB and β-catenin in order to define characteristic features of these lesions. While HID-AB staining clearly allowed for detection of the dysplastic status of the lesions by indicating loss of goblet cells and mucus production (Figure 2h and k), MB staining did not do anything other than stain the dysplastic crypts a darker blue than normal crypts (Figure 2g and j). Swiss roll immunohistochemistry showed β-catenin accumulation in sections coinciding with MB and HID-AB lesions, indicating Wnt signaling pathway activation in both lesions (Figure 2i and l).

Non-corresponding flat ACF and MDF. The one lesion identified as MDF, but not scored as flat ACF, was obviously overlooked since it displayed all characteristics of flat ACF, in particular compressed pit pattern. The 7 lesions that were scored as flat ACF, but not identified as MDF (see Figure 1c), were attempted to be identified in cross sections without success.

Discussion

In the present work, in order to refine the method of detection of flat ACF and to further characterize and define them as early lesions of colon carcinogenesis, we used the novel A/J Min/+ mouse model, that demonstrates extensive spontaneous initiation, promotion and progression of colorectal cancer (14).

Although flat ACF have been recognized as suitable preneoplastic colonic lesions (19-21), few other groups have used them in experimental studies. This is apparently due to difficulties with technical or methodological aspects of flat ACF detection; consequently, the present work aimed at providing more detailed and refined methods of detection. Of particular importance were the time requirements during MB staining: the colons should not be stained for more than 8-10 sec; after which the colons must be stored in 10% formalin or 70% ethanol for at least 24 h. After 24 h, the flat ACF had retained more MB than the surrounding normal epithelium, which, then, allowed for a color distinction between dysplastic and healthy crypts (Figure 1a). The reason for this is unknown; however, Ochiai *et al*. (22) suggest that dysplastic tissue may be more resilient to decolorization after treatment with methanol than normal tissue. This may also potentially be true for decolorization in formalin or 70% ethanol, which is used in the method described here. Other than showing slightly darker coloring in dysplastic crypts, staining histological sections of flat ACF with MB did not provide any additional information to explain why a color difference is produced. Since other structures, such as lymphoid aggregates, may attain similar coloration as flat ACF, an additional feature for flat ACF detection was defined. Thus, in order to classify flat ACF, the lesions must contain compressed crypt openings with gyrus-like pit patterns. The majority of flat ACF did not protrude above the surrounding mucosa; however, a few lesions did appear slightly polypoid. By examining pit patterns, these polypoid flat ACF could be distinguished from classic ACF, a type of lesions that accounts for only 0.4% of the preneoplastic lesions in this model (14).

A high correspondence (93%) between flat ACF and MDF was found and histological characterization of the lesions strongly suggested that these surface biomarkers indeed represent the same dysplastic lesions formed in *Apc*-driven colon carcinogenesis. Hence, the A/J Min/+ mouse model resembles the Pirc rat (23). Of the non-corresponding lesions, one MDF lesion was not scored as a flat ACF lesion. However, that lesion was presumably a flat ACF lesion since the MDF clearly showed all characteristics required for classification as a flat ACF lesion. There were also seven lesions that were scored as flat ACF but not as MDF. Why these lesions were scored as flat ACF while failing to fulfill the criteria for MDF is uncertain. Although the A/J Min/+ mouse only has a very small fraction of classic ACF (14), one explanation may be that some of these non-coincidental lesions were in fact classic ACF, which do not exhibit complete mucin depletion (4). Another potential explanation is that the flat ACF scored may have been a collection of smaller flat ACF in close proximity (rather than one large lesion) with healthy, mucin-producing crypts interspersed between these smaller flat ACF, masking mucin depletion in the adjacent lesions (Figure 1c). It is also possible that non-coincidental lesions represent something that has yet to be described. Unfortunately, preparing histological cross-sections of this small fraction of lesions was unsuccessful.

In AOM-treated B6 Min/+ mice, the correspondence between flat ACF and MDF has been previously determined to be only 57% (13), in contrast to 93% in the present study. The discrepancy between these two studies may be due to additional tumor-causing mechanisms in AOM-treated Min/+

mice that are not present in the spontaneous tumorigenesis of the A/J Min/+ mouse. In the AOM-treated B6 Min/+ mouse (13), some MDF were scored as classic ACF, while in the A/J Min/+ mouse classic ACF are rarely observed (14). In the study by Femia *et al*. (13), some of the lesions scored in the MB-stained colon may have actually been polypoid flat ACF wrongly scored as classic ACF. As the present work clearly shows, flat ACF may appear polypoid (Figure 1b, middle). Here, when these polypoid flat ACF were restained with HID-AB, mucin depletion was unmistakable.

In conclusion, flat ACF can easily be detected by surface examination of MB-stained colons from the A/J Min/+ mouse, which demonstrates extensive spontaneous colon carcinogenesis. The virtually complete overlap between flat ACF and MDF, as well as their comparable histological characteristics, suggests that these structures indeed represent the same early, dysplastic colonic lesion. Consequently, flat ACF are reliable surface biomarkers of *Apc*-driven colon carcinogenesis.

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