Flat Colorectal Cancers Are Genetically Determined and Progress to Invasion without Going through a Polypoid Stage

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Abstract
Growing evidence suggests that flat colorectal cancers (CRC) account for 10% to 20% of all CRCs and that these are frequently associated with more advanced pathologies. However, controversy exists as to the origin and progression of flat CRCs compared with the more common polypoid-type morphology. We report using the azoxymethane mouse model for human CRC that KK/HIJ and 1/LNJ mice develop different frequencies of flat and polypoid tumors; 83% of colon tumors in 1/LNJ mice are flat compared with only 19% in KK/HIJ mice, indicating a strong genetic predisposition to the development of specific CRC morphologies. Like polypoid tumors, all flat tumors show a significant increase in the level of nuclear β-catenin (CATNB1), supported by similar frequencies of mutations in the phosphorylation domain–coding region (codons 32–41) of Catenbl. However, in contrast to previous reports, tumors bearing higher “oncogenic potential” do not cluster in codon 41 of Catenbl. There are no differences between flat and polypoid tumors in the frequency of mutations in codons 12 and 13 of Kras or codon 624 of Braf. Similarly, there are no differences between tumor morphologies in their location along the proximal-to-distal colonic axis or in the relative quantity of intratumor stromal myofibroblasts as marked by the expression of α-smooth muscle actin. Using a combination of serial colonoscopic and histologic analyses, we definitively show that flat CRCs do not develop de novo but progress through a flat adenomatous stage to invasive carcinoma without transit through an intermediary polypoid stage. [Cancer Res 2007;67(24):11594–600]

Introduction
Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States and accounts for the second most common cause of cancer-related deaths with ~145,290 new cases and 56,290 deaths annually (American Cancer Society, 2005). Although advances have been made in the treatment of CRC, the most effective means of lowering cancer incidence is through prevention and early detection (American Cancer Society, 2005). Although colonoscopy is currently the most accurate and widely used method for the detection and removal of colonic neoplasms, there is growing evidence that CRCs with flat morphologies are more difficult to detect and are missed at higher rates, which may contribute to the high incidence of advanced CRC associated with flat morphologies (1–4).

Flat CRCs were first described nearly 30 years ago in Japan, and until recently, have been considered less common in western countries (5–8). However, recent studies have shown that flat adenomas may comprise as many as 22% of colorectal tumors in the United States (6). The discrepancy in the perceived frequency of flat CRCs has also contributed to contradictory hypotheses about the origin and progression of flat CRCs. Supporters of the adenoma-carcinoma sequence posit that flat carcinomas originate through an adenoma intermediate, either by ulceration of a polyp or through the formation of a flat adenoma (9, 10). Others have asserted that the identification of flat carcinomas with no observable adenoma component suggests that flat carcinomas arise de novo without progressing through an adenomatous intermediate (2, 11, 12).

To resolve the molecular and morphologic development of flat CRCs, we molecularly analyzed azoxymethane-induced flat and polypoid tumors and performed serial endoscopy of azoxymethane-induced tumors arising in 1/LNJ and KK/HIJ mice, which predominantly develop flat and polypoid tumors, respectively. Here, we show that there is a genetic predisposition for CRC morphology and that flat carcinomas have polypoid-type mutational spectra and histologic characteristics but originate as flat adenomas, which have a lateral growth pattern without progressing through a polypoid stage.

Materials and Methods
Mice. KK/HIJ and 1/LNJ mice were obtained from The Jackson Laboratory. Mice between 2 and 4 months of age were injected i.p. with 10 mg/kg body weight azoxymethane (Sigma-Aldrich) once a week for 4 or 6 weeks as previously described (Fig. 1). Mice were housed in ventilated racks and provided 5010 chow (LabDiet) and water ad libitum in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility. 1/LNJ mice were euthanized by CO2 asphyxiation 6.5 months after the first azoxymethane injection. Due to the development of large tumors causing intestinal obstruction, KK/HIJ mice were sacrificed ~5 months after the first azoxymethane injection. All experiments were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Necropsy and tumor histology. Upon sacrifice, colons were removed from the cecum to the rectum, gently flushed with PBS, placed on filter paper, and splayed longitudinally. Tumors were counted and diameters measured in their maximum dimension. Swiss rolls were prepared by gently rolling the splayed colons from the rectum to the cecum, pinned with a 30-gauge needle, fixed in 10% neutral-buffered formalin overnight and paraffin-embedded before cutting 7-μm sections. For histologic analysis, sections were stained with H&E and photographed. Sections near the center of each tumor were photographed and analyzed for both cross-sectional area as well as net tumor height using Image J (NIH). The height of cross-sectioned tumors (with tumor base present) was determined by measuring

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perpendicularly from the muscularis propria to the lumen surface. Quantitative measurements were used to define the morphologic class for each tumor. Polypoid tumors were defined as those whose heights were greater than one-half their diameter, whereas flat tumors had heights less than one-half their diameter.

Tumors were classified as low-grade dysplasia if they had a mildly disorganized epithelium lined by hyperchromatic cells with nuclear pseudostratification. Features used to classify tumors with high-grade dysplasia included back-to-back glands, high nuclear to cytoplasmic ratios, loss of cellular polarity, and increased nuclear pleomorphism. Tumors were scored as invasive when there was obvious penetration of dysplastic glands through the muscularis mucosa with associated desmoplastic stromal response.

**Mutation analysis.** Tumor epithelium from H&E-stained tumor sections were isolated using laser-assisted microdissection on a P.A.L.M. system (Zeiss MicroImaging) before isolating DNA with the PicoPure DNA Extraction Kit (Arcturus). PCR was performed to amplify the segment of genomic region coding for the phosphorylation domain of *CATNNB1* (5′ GCTGACCTGATGGAGTTGGA and 3′ GCTGACCTGATGGAGTTGGA), exon 1 of *Kras* (5′ GCCTGCTGAAAATGACTGAG and 3′ CCTCGTTAGGGTGTCGTAC), and exon 624 of *Braf* (5′ TTCTTTTACTCTGGACCTCAGA and 3′ AAGCCTTCACTGGATTCTCG 3′). PCR products were gel-purified and directly sequenced by the University of North Carolina Genome Analysis Facility.

**Immunohistochemistry.** Tissue sections were deparaffinized in xylene and hydrated through a graded alcohol series. Immunohistochemistry to detect *CATNNB1* was done using a mouse anti-*CATNNB1* antibody diluted 1:100 (Transduction Laboratories) with the MOM kit (Vector Laboratories) and visualized using 3,3′-diaminobenzidine (Zymed Laboratories). Primary antibody incubations were performed overnight at 4°C.

**Endoscopy.** Endoscopy was performed between 9 and 26 weeks after azoxymethane injection (Fig. 1). Direct visualization of colonic tumors in vivo was performed using a "Coloview system" (Karl Storz Veterinary Endoscopy). Mice were supplied with food and water until the endoscopy was performed. If fecal material obstructed the view of the endoscope, colonos was flushed with 0.9% saline. For the colonoscopies, the mice were anesthetized with 1.5% to 2% isoflurane and –3 to 4 cm of the colon from the anal verge until the splenic flexure was visualized after inflation of the colon with air. The colonoscopic procedures were digitally recorded on an AIDA Compaq PC.

**Results**

**Genetic background influences colon tumor morphology.** We previously reported that significant differences exist in gross tumor size and morphology among azoxymethane-induced tumors in mouse strains with tumors from KK/HIJ being 1.3-fold larger in average diameter than tumors from I/LNJ.\(^8\) Morphometric analysis of H&E stained sections from KK/HIJ and I/LNJ mice validated these results (data not shown) and also reveal that azoxymethane-induced colorectal tumors recapitulate two morphologic growth patterns seen in human CRCs, outward growing polypoid and lateral growing flat (also called nonpolypoid) cancers (refs. 5, 13, 14; Fig. 2A). To assess the influence of genetic background on tumor morphology, we quantified the number of tumors from each morphologic class in azoxymethane-treated KK/HIJ and I/LNJ mice (Fig. 2B). We found that KK/HIJ mice develop a significantly higher proportion of polypoid tumors (26 of 32) than I/LNJ mice, which predominantly develop flat tumors (19 of 23; \(P = 0.0001\)).

To more accurately compare the sizes of polypoid and flat tumors, we determined the cross-sectional area at the midpoint of each tumor as a measure of maximal cross-sectional area. Polypoid tumors (average area, 3.9 mm\(^2\)) showed a 3.4-fold increase over flat tumors (average area, 1.14 mm\(^2\)) in maximal cross-sectional area \((P = 0.0001; \text{Fig. 2B})\), similar to the difference observed for the height to width (H/W) ratio (polypoid tumors had a 3.2-fold larger average H/W ratio than flat tumors). However, because the two morphologic types occur preferentially in different strains, these comparisons could represent strain differences rather than morphologic differences in growth. To distinguish these possibilities, we performed an interstrain comparison of flat and polypoid tumors separately and found that KK/HIJ and I/LNJ mice do not differ significantly for either tumor morphology (Fig. 2C). These data show that tumor morphology is strongly influenced by strain, whereas tumor growth (size) is associated with the type of morphology and not directly by strain.

**Flat and polypoid tumors have similar mutational spectra.** More than 80% of human CRCs have mutations in the *APC* gene (15), and *CATNNB1* mutations, mutually exclusive with those in *APC*, occur in nearly 10% of human CRCs (16). Conversely, previous analyses of azoxymethane-induced mouse polypoid colorectal tumors have reported frequent mutations in *Catnnb1*, although no mutations were observed in *Apc* (17). The *Catnnb1* mutations occurring in azoxymethane-induced tumors cluster around codons 32 to 34, which codes for a portion of the phosphorylation domain of *CATNNB1* that is required for ubiquitin-mediated degradation (18). A subset of azoxymethane-induced tumors with mutations in

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\(^8\) A.C. Bissahoyo et al., submitted for publication.
codon 41 are reportedly associated with increased oncogenicity in rats (19).

To determine whether differences in tumor morphology are associated with different transforming mutations, we compared the distribution of Catnnb1 mutations between strains and morphologies, all of which show stabilization and nuclear localization of CATNNB1 (Fig. 3A). Of the KK/HIJ tumors analyzed for the presence of Catnnb1 mutations, 17 of 21 (81%) contained mutations in codons 32 to 41 of Catnnb1, similar in frequency and distribution to those observed in the I/LNJ tumors with 16 of 17 (93%) containing Catnnb1 mutations and indicating a strain-independent frequency of Catnnb1 mutations. Similarly, 16 of 18 (89%) polypoid tumors contained Catnnb1 mutations compared with 12 of 14 (86%) flat tumors, suggesting that differences in Catnnb1 mutation frequency or distribution do not underlie morphologic differences (Fig. 3B). In contrast to a previous report stating that codon 41 mutations in Catnnb1 are associated with greater "oncogenic potential" (19), we did not observe a correlation between the distribution of Catnnb1 mutations and oncogenic potential (Fig. 3C). If anything, codon 41 mutations in Catnnb1 are less frequently associated with higher-grade tumors than those in codons 32 to 34.

Although mutations in Kras are frequently associated with more progressed colorectal tumor pathology (15, 20–27), previous reports are conflicting on whether flat tumors contain fewer Kras mutations than polypoid tumors (21, 22). Because the vast majority of reported Kras mutations occur in codons 12 or 13 (25), which encodes the GTP-hydrolysis domain, we analyzed these codons for Kras mutations. Only one Kras mutation (exon 13) was found in 15 polypoid tumors analyzed (7%) and one Kras mutation (exon 12) was found in 15 flat tumors (7%), occurring in KK/HIJ and I/LNJ, respectively. Mutations in BRAF, coding for a signal mediator downstream of KRAS and mutually exclusive with KRAS mutations are associated primarily with serrated adenomas of the colon (28–31). Consequently, we assessed the mutational status of Braf codon 624, corresponding to the reported BRAF codon 599 mutations in human serrated CRCs in polypoid and flat tumors. No Braf mutations were found in any of the tumors analyzed.

Growth pattern or mutational spectra are not associated with stromal expansion. Colorectal adenomas are frequently associated with the proliferation of cancer-associated stroma, which can function as a source of growth signals (32, 33), and in theory, modulate the pattern of tumor growth. Immunostaining of polypoid and flat tumor sections for α-smooth muscle actin (ACTA2), a marker of myofibroblasts, showed subsets of tumors from both morphologies with extensive stromal expansion (Fig. 4), indicating that differences in growth patterns between polypoid and flat tumors are not due to differential expansion of the tumor-associated myofibroblasts. Furthermore, there was extensive stromal expansion in tumors with wild-type Kras and Braf.

**Figure 2.** Morphometric analysis reveals differences in tumor physical variables. A, representative examples of polypoid (magnification, ×1.6; bar, 1 mm) and flat (magnification, ×5; bar, 1 mm) gross tumor histology. B, height, width, and area measurements taken in cross-sections near the center of each tumor reveals that tumors from KK/HIJ have a greater height-to-width ratio than tumors from I/LNJ, which corresponds to differences between polypoid and flat tumors. The ratio of height-to-width is expressed as log2. C, interstrain comparisons between polypoid and flat height-to-width ratios show strain-independent differences in polypoid and flat tumor size.
suggesting that tumors could acquire stromal hyperproliferation without mutations in the KRAS pathway.

Flat invasive carcinomas arise through flat adenoma intermediates. Some reports have suggested that flat colorectal carcinomas develop either through a polypoid intermediate by ulceration and sloughing off of a polyp or formation of a flat adenoma intermediate (9, 10). However, contrasting models have posited that flat carcinomas arise de novo without an adenomatous intermediate (2, 11, 12, 34, 35). To distinguish between these conflicting models, we used endoscopy to visualize and serially follow tumor growth patterns from initiation through progression to invasive carcinoma. Polypoid tumors were first observed as small but notably raised lesions that tended to grow outward (vertically) into the lumen (Fig. 5A). Conversely, flat tumors first appeared as slightly raised, lateral (horizontally) growing lesions with a central depression that over time became larger but remained flat (Figs. 5B and 6A). Polypoid tumors tended to increase in size more quickly than flat tumors. The polypoid and flat tumor morphologies observed with endoscopy were consistent with the histologic characterizations.

No flat tumors were observed that progressed to a polypoid morphology nor did we observe evidence of ulceration in vivo (Fig. 6A). Histologic analysis revealed that flat tumors are capable of progressing to invasive carcinoma, and in all cases, adenomatous components were observed (Fig. 6B and C). Taken together, these data indicate that flat invasive carcinomas developing in the azoxymethane model do not arise de novo, but rather, through a flat adenoma intermediate without ever exhibiting a polypoid morphologic stage. Furthermore, no significant differences exist between polypoid and flat morphologies in the proportion of invasive carcinomas (polypoid, 17%; flat, 15%).

Discussion

There is an increasing awareness that flat CRCs, originally described in Japan, occur in western countries. Recent reports show that the prevalence of CRCs with flat morphologies are not geographically constrained and are generally underappreciated due to the greater difficulty of their detection during routine colonoscopies (1, 4, 6). Using the azoxymethane mouse model of human CRC,
we show that genetic background strongly influences the morphologic growth pattern of CRCs, independent of the initiating mutations. We found that azoxymethane-treated mice develop two predominant morphologic varieties of colorectal tumors, which show similarity to human CRCs with polypoid and flat morphologies. Azoxymethane predominantly induces large, outward-growing polypoid tumors in KK/HIJ mice, whereas in I/LNJ mice, azoxymethane predominantly induces smaller, lateral growing flat tumors, supporting the importance of genetic background in determining the growth pattern of CRCs. Interestingly, polypoid tumours have a higher risk of developing into invasive carcinomas compared to flat ones. This is illustrated in Figure 6, which shows a flat adenoma progressing to invasive carcinoma. The histology of the invasive carcinoma is characterized by malignant glands invading through the muscularis mucosa with mild stromal desmoplasia and pools of submucosal mucin. This progression highlights the importance of genetic background in the development and progression of CRCs.
and flat tumors have a similar mutational spectra with equivalent Catnb1 and Kras mutation frequencies and an absence of Braf mutations, indicating that differential initiating mutations are not the underlining mechanism driving morphologic growth. Similarly, we observed extensive growth of ACTA2-positive stroma in a subset of both polypoid and flat colorectal tumors uncorrelated with Catnb1 and Kras mutation status, indicating that myofibroblast expansion is also not responsible for the differential growth patterns. The predominant initiating mutations in human CRCs result in the inactivation of APC with consequential stabilization of CATNNB1 (reviewed in ref. 36). However, up to 50% of human CRCs that have no detectable APC mutations contain CATNNB1 mutations resulting in ubiquitination-resistant, stabilized CATNNB1 protein (16). Consistent with previous reports (15, 19), we found that Catnb1 mutations tended to cluster around codons 32 to 34, which codes for a portion of the phosphorylation domain of the mature protein. Previous data have suggested that extended dimethylhydrazine, a precursor to azoxymethane, treatment results in a higher proportion of tumors with Catnb1 mutations in codon 41 as opposed to codons 32 to 34 (19). The conclusions offered to explain these results were that extended carcinogen treatment results in a higher incidence of mutations in codon 41, which normally account for a minority of mutations observed with shorter carcinogen treatment, but conferring higher "oncogenic potential." Contrary to this theory, we found no correlation between the site of Catnb1 mutation and tumor oncogenicity. Based on the differential oncogenicity model, one would expect that tumors bearing codon 41 mutations would have a tendency toward more advanced stages. Yet, we did not observe tumors with codon 41 mutations as being any more advanced than those with mutations in codons 32 to 34. Although the former study was performed in rats, this difference is unlikely a species-specific difference because both rats and mice have similar distributions of Catnb1 mutations after 5 weeks of dimethylhydrazine treatment (19). An alternative possibility is that mutations in codon 41 of Catnb1 render cells more resistant to cell death caused by additional carcinogen treatment. Significant attention has been paid to the role of secondary mutations in human CRCs, particularly KRAS (20, 21, 24, 37, 38). The frequency of Kras mutations we detected in azoxymethane-induced flat tumors is similar to that observed in polypoid tumors and is consistent with findings for human flat tumors (21, 24, 26). However, the relatively low frequency of Kras mutations in azoxymethane-induced polypoid tumors contrasts with that reported in human polypoid tumors (20, 25, 26). Vogelstein and coworkers proposed that Kras mutations were significantly more common in larger tumors (37). Based on the great variability in size of non-Kras mutant tumors, our data suggests that tumor size variations occur independent of Kras mutation status.

There has been considerable controversy over the origin of flat invasive colorectal carcinomas of the colon. Although some models predict that flat carcinomas form through the adenoma-carcinoma sequence, including a polypoid stage with subsequent ulceration, other models predict a de novo pathway whereby flat CRCs arise directly as carcinomas without an adenoma intermediate. Here, we used serial endoscopy and histology to visualize the initial formation and subsequent progression of polypoid and flat colorectal tumors that arise in the azoxymethane mouse model of human CRC and show that flat invasive carcinomas do not arise de novo, but are preceded by flat adenomas without progression through a polypoid stage, consistent with original reports describing human flat CRCs (5, 39).

In this study, we combined the use of the azoxymethane mouse model of human CRC with endoscopy to directly visualize the initiation and progression of flat and polypoid tumors in vivo. Our results show that flat and polypoid colorectal tumors are distinct entities, despite having similar mutational spectra. One explanation for this might be due to the presence of as yet unknown strain-specific genetic modifiers that influence tumor shape or growth pattern. Furthermore, it is plausible that host-dependent growth patterns are manifest through tumor nonautonomous mechanisms like strain-specific differences in the colonic stromal environment. Because CRCs with flat morphologies are under-detected in western societies during routine colonoscopies, the azoxymethane model reported here can support investigations into the unique molecular characteristics of flat CRCs and should aid in the development of new methods for their detection, prevention, and treatment.

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