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/HJ and I/LNJ mice develop different frequencies of flat and polypoid tumors; 83% of colon tumors in I/LNJ mice are flat in comparison with only 19% in KK/HJ 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 Catnb1. However, in contrast to previous reports, tumors bearing higher “oncogenic potential” do not cluster in codon 41 of Catnb1. 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.

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 I/LNJ and KK/HJ 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/HJ and I/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. I/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/HJ 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 ImageJ (NIH). The height of cross-sectioned tumors (with tumor base present) was determined by measuring...
**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. 24). 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. The results (Fig. 2A) reveal that azoxymethane induced colorectal tumors recapitulate two major morphologic growth patterns seen in human CRCs, outward growing polypoid and lateral growing flat (also called nonpolypoid) cancers (refs. 5, 13, 14). These patterns are similar to the difference observed for the height to weight (H/W) ratio of polypoid tumors having 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 **Catnb1**, although no mutations were observed in **Apc** (17). The **Catnb1** 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.

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**Figure 1.** Experimental timeline for azoxymethane-induced CRC induction and analysis. Tumor induction for initial histologic studies was done with four weekly azoxymethane injections (black arrowheads), whereas colonoscopy and progression analyses were done using six weekly azoxymethane injections (black plus gray arrowheads). Endoscopy was performed from 9 to 20 wk after the first azoxymethane injection (white arrowheads). Due to large tumors resulting in intestinal obstruction, KK/HIJ mice were sacrificed at 20 wk rather than 26 wk post-azoxymethane induction.
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 through 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 tumors observed in vivo through serial colonoscopy.

**Figure 5.** Growth and progression of polypoid and flat tumors observed in vivo through serial colonoscopy. 

A, KK/HIJ mouse at week 17 with three polypoid tumors (white arrows) and at week 20 with the same cluster of three polypoid tumors plus an additional new polypoid tumor (black arrow). B, I/LNJ mouse at week 17 with a single flat tumor containing a central depression (black arrow) and at week 20 with three flat tumors (white arrows) in the same location as week 17.

**Figure 6.** Flat carcinomas arise through a flat adenoma intermediate. 

A, no visible tumors during an initial colonoscopic examination at week 9 and a single flat tumor in the same mouse at week 12 (shiny area in week 9 image is fecal matter). B, representative flat invasive carcinoma arising from a flat adenoma (magnification, ×1.6; bar, 1 mm). C, enlargement of invasive carcinoma in B, characterized by malignant glands invading through the muscularis mucosa (arrow) with mild stromal desmoplasia and pools of submucosal mucin (arrowheads; magnification, ×10; bar, 100 microns).
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 underlying mechanism driving morphologic growth. Similarly, we observed extensive growth of ACTA2-positive stroma in a subset of both polyoid 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 consequent stabilization of *CATNB1* (reviewed in ref. 36). However, up to 50% of human CRCs that have no detectable *APC* mutations contain *CATNB1* mutations resulting in ubiquitination-resistant, stabilized *CATNB1* 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 polyoid tumors and is consistent with findings for human flat tumors (21, 24, 26). However, the relatively low frequency of *Kras* mutations in azoxymethane-induced polyoid tumors contrasts with that reported in human polyoid 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 polyoid 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 polyoid 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 polyoid 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 polyoid tumors in vivo. Our results show that flat and polyoid 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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