Clonal Structure of Carcinogen-Induced Intestinal Tumors in Mice

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Abstract
Previous studies have shown that intestinal tumors from Apc<sup>Min+/−</sup> (Min) mice and familial adenomatous polyposis (FAP) patients are often polyclonal. We sought to determine whether polyclonality is unique to tumors arising from hereditary predispositions or, instead, is a common feature of intestinal tumorigenesis in other pathways to tumorigenesis. Ethylnitrosourea-induced intestinal tumors from mice wild type at the Apc locus and chimeric for the Rosa26 lineage marker were analyzed. Many were overtly polyclonal, being composed of a mixture of Rosa26<sup>+</sup> and Rosa26<sup>−</sup> neoplastic cells. Statistical analyses revealed that polyclonality could be explained by interactions between two initiated clones separated by a very short distance. The frequency of overtly polyclonal tumors and the range of interactions estimated in this model are similar to those observed when analyzing familial tumors from Min mice. Thus, polyclonality does not depend on the familial pathway to tumorigenesis. Interactions between two initiated clones might provide a selective advantage during the early stages of intestinal tumorigenesis. Cancer Prev Res; 4(6): 916–23. ©2011 AACR.

Introduction
The clonal origin of intestinal tumors is an active area of investigation. Tumors could be monoclonal, being derived from a single progenitor, or alternatively, tumors could be polyclonal, being derived from 2 or more progenitors. Evidence for polyclonality has been steadily accruing over many years (1–6). Novelli and colleagues analyzed benign colorectal adenomas from patients who carried a germ line mutation in the adenomatous polyposis coli (APC) gene and who was an XY/XO mosaic (3). Some tumors were overtly polyclonal, being composed of both XY and XO cells. Critics argued that this study did not prove that intestinal tumors could be polyclonal because the karyotype was inherently unstable. Thirlwell and colleagues analyzed benign colorectal adenomas from patients with FAP for somatic mutations in APC (6). All tumors were polyclonal with adjacent dysplastic crypts carrying different mutations. Thus, the findings from these 2 studies indicate that familial tumors can be polyclonal.

Mouse models are a tool to better understand the clonal origin of tumors in the mammalian intestine. Merritt and colleagues analyzed benign intestinal adenomas from C57Bl/6 (B6) mice that were carrying the Min allele of the Apc gene and chimeric for Rosa26 expression (4). Several tumors were composed of Rosa26<sup>+</sup> (blue) and Rosa26<sup>−</sup> cells (white). In this model, genetic instability was not an issue; clearly, familial tumors can be polyclonal. The authors offered 2 different mechanisms to explain polyclonality: random collision between 2 or more existing tumors and clonal interactions (4). These explanations could not be distinguished because the tumor multiplicity was extremely high (3, 4). Thliveris and colleagues analyzed tumors from B6 Apc<sup>Min+/−</sup> (Min) mice that were homozygous for the resistance allele of Mom1 and chimeric for Rosa26 expression (5). Mom1 is a semidominant polymorphic modifier that reduces the multiplicity and size of intestinal tumors in Min mice (7); 1 component of this locus is Pla2g2a, which encodes a secretary phospholipase (8, 9). Many early adenomas in these aggregation chimeras were overtly polyclonal even though the probability of 2 tumors coalescing was significantly reduced. These findings support the hypothesis that polyclonal tumors arise from clonal interactions. Statistical analyses of the chimism revealed that polyclonality could be explained by interactions between 2 or 3 initiated clones occurring over a very short distance (5, 10).

Is polyclonality peculiar to humans and mice with a genetic predisposition to intestinal cancer? Investigators...
have sought to determine the clonality of intestinal tumors from mice treated with a carcinogen. Ponder and Wilkinson analyzed colon lesions from CBA/Ca ↔ B6 aggregation chimeras treated with azoxymethane (11). All dysplastic lesions (55 of 55) and the majority of adenomas (15 of 17) that developed were composed of cells derived from a single progenitor. Tatematsu and colleagues analyzed colon lesions from C3H/HeN ↔ BALB/c aggregation chimeras treated with 1,2-dimethylnitrosourea (12). The majority of adenomas (90 of 91) and the majority of adenocarcinomas (107 of 119) were monoclonal. The authors argued that the few heterotopic tumors that did form appeared to arise from the random collision because cells from different lineages were arranged in discrete areas rather than intermingled. These 2 studies had the following limitations: (i) the aggregation chimeras were composed of cells with significantly different susceptibilities to the carcinogen (e.g., CBA mice treated with azoxymethane developed 9 times more lesions than B6 mice) and (ii) patch size was large so individual tumors would be homotypic even if derived from multiple progenitors. Griffiths and colleagues analyzed colon lesions from C3H mice mosaic for the expression of glucose-6-phosphate dehydrogenase (13). Colon were rolled, sectioned at 8 to 10 levels, and stained. The majority of the tumors (27 of 28) were composed of cells derived from a single progenitor. The authors, however, acknowledged that they could not rule out that a small percentage of tumors are derived from multiple progenitors because only 20% of crypts were adjacent to crypts with a discordant phenotype. To exclude the possibility that 5% of tumors were polyclonal would have required several hundred tumors to be analyzed.

In this study, we determined the clonality of intestinal tumors in B6 mice treated with ethylnitrosourea (ENU). One of the methods that we employed involved sectioning each tumor in toto. Unlike previous studies, we found that many carcinogen-induced tumors can be overtly polyclonal. Statistical analyses were conducted to assess likely explanations for the polyclonality, the range of clonal interactions, and the number of contributing clones.

Methods

Mouse strains and generation of aggregation chimeras

B6 mice and B6 mice heterozygous for the Rosa26 transgene expressing lacZ were obtained from The Jackson Laboratory. Stocks of these strains were maintained by continually backcrossing with B6/J mice (The Jackson Laboratory) that were imported every fifth generation. Offspring were screened for the Rosa26 transgene using PCR assays of DNA isolated from toe clips. Aggregation chimeras were generated by fusing embryos together from 2 crosses: B6 Apc+/+ × B6 Apc+/+ and B6 Apc+/+ × B6 Apc+/+ Rosa26−. Ear snips were also taken from each animal and stained with X-Gal (US Biological) to ascertain β-galactosidase chimerism. Apc+/+ ↔ Apc+/+ Rosa26− mice were found at the expected ratio.

B6 and B6 Apc+/+ ↔ Apc+/+ Rosa26− were treated with ENU to induce intestinal tumors. Pregnant females, neonates, or adults received a single intraperitoneal injection. The dose was 250 µg/g of body weight. The treated mice were allowed to age until moribund.

Staining and serial sectioning protocol

Whole mounts of the intestines were prepared and stained with X-Gal as previously described (5). Sections were then postfixed in 10% formalin overnight and stored in 70% ethanol. The full intestinal tract was photographed and tumors were excised. All adenomas were embedded in paraffin, serially sectioned in toto, and arrayed as two 5-µm sections per slide. Every tenth slide was counterstained with hematoxylin and eosin. When a more complete analysis was required, additional slides were counterstained with nuclear fast red. A homotypic tumor could be composed of cells from a single progenitor or else from cells from multiple progenitors with the same Rosa26 status, whereas a heterotypic tumor is overtly polyclonal.

Analysis of chimerism

The pattern of chimerism, which is the size and shape of blue and white patches displayed in the intestine, affects our ability to observe overtly polyclonal tumors. To quantify these patterns, each binary image of the intestine was converted to a distance map image, which records for each pixel the distance to the closest pixel of opposite color. Such maps were then summarized by 2 functions; 1 for each color. Each function is a cumulative distribution function of the distances associated with the specific color, documenting for each distance the proportion of blue (or white) pixels whose distance map value is no longer than that distance. The distance map functions carry substantial information about the pattern of chimerism and provide more information about the size of blue and white patches than other statistical approaches. Computations were done using the R package EBImage (14) and custom R code (available on request from the authors: www.r-project.org).

The proportion of blue pixels present in each of the mucosal images gave an overestimate of the underlying blue proportion owing to imaging artifacts (e.g., blue villi overlaying white crypts increase the estimated proportion blue); but, white villi, being translucent, do not decrease the estimate if they overlay blue crypts. To account for this effect, we adjusted the observed blue proportions using a simple linear regression model that was fit to data from 5 sections on which both mucosal and serosal images were available. We found that the corrected proportion of blue was equal to 0.776 multiplied by proportion of blue that was observed on the mucosal side minus 0.0119.

Statistical evaluation of the random collision hypothesis

To determine whether the rate of overtly polyclonal tumors was consistent with the coalescence of tumors that originate independently and uniformly throughout the intestine, the statistical test of Newton and colleagues...
was applied (10). This test computes a posterior predictive $P$ value for each chimeric mouse, measuring the probability associated with the number of overtly polyclonal tumors, computed under the assumption of uniform random collision.

**Statistical evaluation of the clonal interactions hypothesis**

The pattern of chimerism described by the distance map functions was combined with tumor phenotype data to address questions about the nature of clonal interaction using 2 different models. In both models, the log likelihood was computed—the logarithm of the probability of the observed phenotypes, taken conditionally on the distance map data (i.e., randomness in the distance maps themselves is not modeled). Contributions to the log likelihood came from each tumor having an unambiguous phenotype; the associated probability was computed from image data specific to the region where that tumor was located. In the disk model (5), the phenotype probability was derived from the distance map function. For example, the probability of a homotypic blue tumor is the probability that an initiated clone is blue and the minimum distance to the nearest white clone exceeds a parameter $\delta$. In the simpler "arbitrary range" model, the probabilities use only the overall proportion of blue and white tissue in each region.

**Identification of Apc mutations**

Tumors were analyzed for Apc mutations using the *in vitro* synthesis of protein as described previously (15). This assay allows truncating mutations between codons 677 and 1,609 to be detected.

**Results**

**Clonality of carcinogen-induced intestinal tumors**

Two different approaches were used to determine the clonal structure of carcinogen-induced tumors. B6 mice were treated at different ages with ENU and allowed to age until moribund. Those treated as fetuses, neonates, and adults developed on average $1 / C6^2$, $5 / C6^4$, and $21 / C6^7$ tumors, respectively (Table 1; Kruskal–Wallis test; $P = 0.0003$). Presumably, this difference in tumor multiplicity reflects the number of cells that could potentially be transformed to initiate tumorigenesis. The clonal structure of each tumor was assessed by scoring the number of Apc mutations using the *in vitro* synthesis of protein assay in which 2 regions of exon 15 were tested for truncations (Table 1 and Fig. 1A; ref. 16). We reasoned that 3 or more truncating mutations in Apc are indicative of a
polyclonal structure, whereas 1 or 2 mutations are indicative of a monoclonal structure. A few tumors (3 of 145) had 3 or 4 mutations. The frequency may be low for 2 reasons: (i) a small portion of the \textit{Apc} gene was tested or (ii) tumorigenesis may be initiated by mutations in genes other than \textit{Apc}. Some tumors (6 of 29) were composed of a mixture of \textit{Apc}-positive and \textit{Apc}-negative neoplastic cells as determined by immunohistochemistry (Fig. 1B and C). As a control, tumors were analyzed from B6 mice carrying the \textit{Min} allele of \textit{Apc} and lacking mismatch repair activity because they often carry truncating mutations. The majority of tumors (5 of 7) from the controls had 2 mutations. These findings indicate that ENU-induced tumors might be polyclonal, but the approach had several limitations and the results are open to alternative interpretations.

We decided to use chimeric mice to determine with more certainty the clonal structure of ENU-induced intestinal tumors. \textit{Apc}^{+/+} ↔ \textit{Apc}^{+/+} Rosa26^- aggregation chimeras that were treated with ENU developed on average 5 ± 4 intestinal tumors with a range from 0 to 9. Many of the tumors (47%) were overtly polyclonal, composed of a mixture of white and blue neoplastic clones as judged by histology in which features indicative of neoplastic transformation were the loss of typical anatomic organization of crypts, in conjunction with cellular atypia of the constituent cells (Table 2 and Fig. 2D and E). Therefore, carcinogen-induced tumors, like early familial tumors, can have a polyclonal structure.

The percentage of overtly polyclonal tumors in ENU-treated mice was significantly higher than that observed in Min mice (4, 5). The probability of observing 9 overtly polyclonal tumors of 19 is only 0.0067 if the underlying rate of overt polyclonality is 22% as reported for familial tumors in Min mice by Thliveris and colleagues in 2005 (5). One possible explanation for the higher percentage of overtly polyclonal tumors could be a different pattern of chimerism, which is the relationship of blue to white tissue that is described in part by assessing the distance between clones derived from the 2 different lineages (Fig. 2F). For example, very small blue and white patches increase the opportunity for comingling of clones of opposite color, even if there is no change in the underlying rate of

### Table 2. Clonal structure of ENU-induced tumors in aggregation chimeras

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**NOTE:** Sections 1 to 4 refer to quarters of the small intestine numbered from proximal to distal; section C refers to the colon. Some tumors were not scorable because of poor fixation, sectioning, or staining.
Tumor phenotype was determined by examining paraffin sections stained with hematoxylin and eosin (D); the outlined area is enlarged in (E). In this example, blue and white neoplastic cells are evident at higher magnification (B) and the digitized image (C). Two tumors were overtly polyclonal (green) and one was not scorable (red). The entire colon from chimera 101 is shown (A); the outlined area is enlarged in (B). Three tumors are evident in the whole mount at higher magnification (B) and the digitized image (C). From blue to white indicates cells are blue. The entire colon from chimera 101 is shown (A); the outlined area is enlarged in (B). Three tumors are evident in the whole mount at higher magnification (B) and the digitized image (C). Two tumors were overtly polyclonal (green) and one was not scorable (red). Tumor phenotype was determined by examining paraffin sections stained with hematoxylin and eosin (D); the outlined area is enlarged in (E). In this example, blue and white neoplastic cells are evident at higher magnification (E). Distance maps describing the relationship between blue and white tissue in the colon of chimera 101 were calculated and summarized by cumulative distribution functions (F). The intestine from each aggregation chimeras was segmented into 5 regions. The pattern of chimerism for each region is summarized by the median of the minimum distance to the opposite color; specifically, the median of the distance map function and compared with the proportion of the reference color (G, red circles). The arrow indicates the data for the colon of chimera 101. Results from very small pieces adjacent to tumors that were used in our previous study involving Min mice (5) are shown for comparison (A and B, white-filled circles) and indicate that the pattern of chimerism is not substantially different between the 2 studies. Note that the wider distribution of samples from the earlier study along the horizontal axis is attributable to size of the very small pieces that were analyzed. D, scale bar = 500 μm.

Polyclonality could result from the random collision of initiated tumors, which would occur when 2 or more growing tumors in close proximity coalesce to form a single tumor. A statistical test of the uniform random collision hypothesis (3) shows that the number of overtly polyclonal tumors in these mice is too high to be accounted for by having clones initiate uniformly at random and coalesce when sufficiently close (Table 2, chimeras 101 and 153, each \( P < 10^{-4} \)). This finding is reasonable because tumor multiplicity was very low in all chimeras treated with ENU.

Another possibility is that polyclonal tumors arise from clonal interactions. By combining tumor phenotype with image data on chimerism, we addressed questions about the possible nature of such interactions. The disk model (1), which is an elementary stochastic model of clonal interaction via recruitment after initiation, allowed us to make a judgment about the spatial extent of clonal interactions. In this model, an initiated clone emerges uniformly at random in the intestine and recruits all clones within distance \( \delta \) to participate in forming a polyclonal tumor. The probability that the resulting tumor is blue, white, or a mixture of blue and white depends on the pattern of the chimerism as informed by distance maps. A tumor is blue only if the initiating clone is blue and the distance to the nearest white clone is greater than \( \delta \). Each tumor phenotype represents a trinomial observation, with the probability determined by the 2 distance maps corresponding to the section in which the tumor formed and \( \delta \). The logarithm of the probability of the observed data, that is, the log likelihood as a function of \( \delta \) was plotted (Fig. 3, curve). The maximum likelihood estimate for \( \delta \) is 68 μm with a 95% CI of 38 to 121 μm. This short distance is roughly the same as the distance between the centers of 2 adjacent crypts.

Number of participating clones

One restriction of the disk model is that it entails full participation, by recruitment, of all cells within a given distance of an initiated clone. We sought to relax this restriction by considering stochastic models in which an initiated clone participates with some number (\( v \)) of partners during tumor initiation. The most elementary model in this direction does not consider interaction range at all and simply posits that a tumor is formed by the interaction (not collision) of \( v + 1 \) clones sampled uniformly at random from the whole intestine. The likelihood is highest when \( v \) equals 1 (Fig. 3). Thus, in the arbitrary range model, the interaction between merely 2 initiated clones is
sufficient to explain the number of overtly polyclonal tumors observed in ENU-treated mice.

Discussion

Polyclonality is a common feature of early adenomas in the mammalian intestine regardless of the pathway to tumorigenesis (refs. 3, 5, 6, 10; this study). We determined the clonal structure of intestinal tumors in mice treated with ENU. Statistical analyses revealed that the high percentage of overtly polyclonal tumors was not consistent with simple random collision of initiated tumors and that it could be explained by interactions between 2 initiated clones or by the interaction of many clones within a very small region. The same conclusion was drawn when analyzing familial tumors from Min mice (5). The fact that this structure is a common feature of adenomas supports the hypothesis that polyclonality provides a selective advantage at least during the early stages of tumorigenesis in the mammalian intestine.

The percentage of overtly polyclonal tumors in ENU-treated mice was significantly higher than that observed in Min mice. If polyclonality thwarts the host response to some degree, its prevalence will depend on advantage afforded and the biological context. In Min mice, every cell in the animal is heterozygous for a mutation in Apc, and this genotype profoundly affects homeostasis in the intestinal epithelium (17). The resulting elevated expression of β-catenin is associated with decreased proliferation, decreased apoptosis, and decreased enterocyte crypt villus migration. These changes might abrogate the need for interaction with other initiated clones during tumor establishment and growth. By contrast, in mice treated with ENU, any single initiated clone is completely surrounded by normal clones and consequently might be unable to become established on its own. Consistent with this hypothesis, Allard and colleagues showed that stationary normal cells caused growth inhibition of SV40-transformed derivatives through cell-cycle arrest (18).

Polyclonality does not depend on the architecture of crypts in the neonatal intestine. When mice are born, crypts are predominantly polyclonal, but around 3 weeks of age, they become monoclonal. The conversion is referred to as crypt purification and is poorly understood. Tumors in Min mice form at a very early age, so one might argue that polyclonality simply reflects the architecture of the neonatal crypt. In this study, we were able to explicitly test this possibility because the timing of tumor initiation was dictated by the age at which mice were treated with ENU. Multiple Apc mutations were observed in intestinal tumors from neonates or adults treated with ENU and overtly polyclonal tumors were identified in Apc+/+ ↔ Apc+/+Rosa26+ adults treated with ENU. Thus, polyclonal tumors can arise when tumorigenesis is initiated in neonatal or adult intestines.

Interactions between 2 clones are sufficient to account for the number of overtly polyclonal tumors observed in Apc+/+ ↔ Apc+/+Rosa26+ adults treated with ENU. However, we cannot exclude the possibility that multiple initiated clones contribute to a single tumor if the interactions occur over a very short distance. Heterotypic tumors often appear to have multiple discrete blue clones (e.g., the large tumor in Fig. 2B and C). To fully understand complexity, 2 different approaches could be used. The ratio of white to blue tissue could be varied by fusing embryos at different stages rather than fusing together only 4- to 8-cell embryos as in this study. Variation in chimerism would allow us to further test the number of participating clones as well as the range of interactions. Alternatively, this issue could be addressed by being able to distinguish multiple lineages, as intestinal tumors might be composed of neoplastic cells from 2, 3, 4, or even more progenitors. When we began this study, Rosa26 was the only lineage marker available, but now a number of different markers are available. Livet and colleagues recently showed that up to 90 different neurons in the brain could be uniquely labeled using a transgene in which the expression of 4 different fluorescent proteins is stochastic (19). This system has been adapted for the intestine (20). Understanding the complexity is fundamental to discerning how polyclonality could affect cancer prevention and therapeutic strategies. Different clones might respond in different ways to treatment and consequently affect the overall response of a tumor.

The nature of the clonal interactions is unknown. An initiated clone could recruit 1 or more neighboring clones. Neoplastic cells within a dysplastic crypt might release
mitogenic factors that affect cellular proliferation in neighboring normal crypts. Indeed, the normal intestinal epithelium adjacent to tumors is often hyperplastic (21). This change could increase the chance that Apc activity is lost in a neighboring crypt. A mutation might occur in Apc because errors during replication are not repaired in the context of high rates of proliferation or else the wild-type allele might be lost by somatic recombination. Alternatively, 2 or more initiated clones might cooperate simply because they are juxtaposed. For example, ENU might transform numerous clones, but only those clones in close proximity would be able to alter the microenvironment in a way that is permissive to tumor formation and growth. These possibilities can be distinguished by generating aggregation chimeras in which the components have different susceptibilities to tumorigenesis and comparing the number of overtly polyclonal tumors to the number predicted from the recruitment model and the cooperation model.

Polyclonality might persist as tumors progress. The most recent studies addressing clonal structure of intestinal tumors in mice and humans have analyzed only early adenomas. Min mice develop on average 100 tumors and live up to 97 days. All tumors in these mice are benign adenomas. In this study, mice treated with ENU developed relatively few tumors and lived up to 300 days. Two tumors in these mice were invasive adenocarcinomas of which 1 was overtly polyclonal. This observation indicates that advanced tumors can be polyclonal. In 1967, Beutler and colleagues found that the primary colorectal tumor and 7 metastatic lesions in the liver of a single patient were heterotypic (22). This finding has been dismissed over the years because the patient developed cancer very early in life. Mouse models are becoming available that will allow persistence of polyclonality during tumor progression to be more rigorously tested. Recently, Hung and colleagues have reported that mice carrying mutations in Apc and KRAS develop colon tumors that metastasize to the liver (23).

This study does have some limitations. First, we were able to follow only 2 lineages in the aggregation chimeras that we generated. New animal models are becoming available which will allow multiple lineages to be followed. Second, it must be recognized that nonuniform but still random collision is difficult to separate from models involving explicit clonal interaction. For example, initiated tumors could cluster spatially owing to some nonuniform field effect; the resulting polyclonality would not have required direct interaction but would have emerged by response to preexisting regions of high tumor potential. Finally, we interrogated the observed heterotypic rates and image data in ENU-treated mice using elementary statistical models of tumor formation to make some judgments about the nature of clonal interaction in polyclonal tumors. We fully appreciate that the statistical models described are extreme simplifications of the dynamic mechanisms truly at work. As has been found in so many other domains, the use of such simplified models provides a language to discuss primary factors affecting the system (24) and puts a sharper focus on the information content of the available data.

Our analysis of polyclonality in carcinogen-induced intestinal tumors has provided the following new insights: (i) polyclonality is common in ENU-induced tumors as well as in tumors arising from hereditary predisposition, (ii) the formation of polyclonal tumors is independent of the crypt architecture, and (iii) statistically, the formation of polyclonal tumors can be explained by the interactions between a pair of clones or even by short range interactions between many initiated clones but not by the accidental coalescence of growing tumors because of uniform random collision. Because polyclonality is common in 2 very different pathways to tumorigenesis, further study is clearly warranted. A full understanding of the clonal origin of tumors has the potential to impact cancer prevention and maybe even therapy in the colon.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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Andrew T. Thliveris, Linda Clipson, Alanna White, et al.


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