Cell, Vol. 87, 159–170, October 18, 1996, Copyright ©1996 by Cell Press

Lessons from Hereditary Colorectal Cancer

Review

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A large body of evidence supports the idea that accumulated genetic changes underlie the development of neoplasia. This multistep process is well illustrated by colorectal cancers, which typically develop over decades and appear to require at least seven genetic events for completion. Even so, inheritance of a single altered gene can result in a marked predisposition to colorectal cancer in two distinct syndromes, Familial Adenomatous Polyposis (FAP) and Hereditary Nonpolyposis Colorectal Cancer (HNPCC). Recent evidence suggests that the genetic defect in FAP affects the rate of tumor initiation by targeting the gatekeeper function of the *APC* gene. In contrast, the defect in HNPCC largely affects tumor progression by targeting the genome guardian function of DNA mismatch repair. Studies of these syndromes have provided unique insights into both inherited and sporadic forms of human tumors.

Introduction

At least 50% of the Western population develops a colorectal tumor by the age of 70, and in about 1 in 10 of these individuals, progression to malignancy ensues. As a result, colorectal cancer is the second leading cause of cancer death in the United States and first when smoking-related cancers are excluded (Parker et al., 1996). Epidemiological studies have suggested that at least 15% of colorectal cancers occur in dominantly inherited patterns (Cannon-Albright et al., 1988; Houlston et al., 1992). The two best defined familial forms are FAP and HNPCC. In the past five years, the genetic bases for both of these syndromes have been discovered, providing new insights into the nature of human neoplasia.

Familial Adenomatous Polyposis

FAP is an autosomal, dominantly inherited disease that affects about 1 in 7000 individuals. Patients with FAP typically develop hundreds to thousands of colorectal tumors (called adenomas or adenomatous polyps) during their second and third decades of life (Figure 1). Although these benign tumors are not individually life-threatening, their large numbers virtually guarantee that some will progress to invasive lesions (called cancers or carcinomas). Additionally, FAP patients often develop extracolonic manifestations, including retinal lesions, osteomas, desmoids of the skin, and brain tumors.

Adenomatous polyposis was first observed in the mid-18th century, and its inherited nature was already recognized by 1900. However, it wasn't until the last decade that its molecular pathogenesis was elucidated. The first clue was a cytogenetically evident interstitial deletion of chromosome 5q in a patient with polyposis (Herrera et al., 1986). This observation stimulated molecular studies that demonstrated tight linkage of the disease to markers on chromosome 5q21 (Bodmer et al., 1987; Leppert et al., 1987). Following the path demarcated by germline alterations in FAP patients and somatic alterations in sporadic colorectal tumors, it became possible to identify the adenomatous polyposis coli (*APC*) gene and to prove that it caused FAP by demonstrating cosegregation of mutant *APC* alleles in affected kindreds (Groden et al., 1991; Nishisho et al., 1991).

Rate-Limiting Events in Tumorigenesis

Patients with germline mutations of *APC* do not necessarily develop colorectal cancer; they simply are at much greater risk to do so than the general population. In order for tumors to form, additional genetic alterations are apparently required. Thus, although FAP patients each develop numerous colorectal tumors, only about 1 of every 10^6 colorectal epithelial stem cells gives rise to such a tumor. Studies in humans with FAP, as well as in mice with analogous mutations of the murine homolog of *APC*, have suggested that the rate-limiting step in tumor initiation is a somatic mutation of the wild-type *APC* allele inherited from the unaffected parent (Ichii et al., 1992; Levy et al., 1994; Luongo et al., 1994). The small fraction of colorectal epithelial stem cells that become neoplastic is in reasonable accord with the low rate of somatic mutation of *APC* expected in normal colon cells. Thus, the study of FAP provides strong support for the "two-hit" hypothesis of Knudson, originally proposed to explain the familial and nonfamilial incidences of childhood tumors such as retinoblastoma and Wilms' tumor (Knudson, 1993). The large numbers of tumors that can be analyzed at early stages in FAP have provided the kind of direct evidence supporting this hypothesis that is difficult to obtain in other tumor types.

Phenotype Versus Genotype

Another general principle illustrated by FAP concerns the complex relationship between genotype and phenotype. FAP patients do not develop uniform clinical features, despite the fact that all have mutations of the same gene and virtually all mutations result in C-terminally truncated proteins (Table 1). In some cases, the difference in phenotype is due to the type of mutation. For example, retinal lesions (congenital hypertrophy of the retinal pigment epithelium, called CHRPE) are associated with truncating mutations between codons 463 and 1387 (Figure 2) (Olschwang et al., 1993). Truncating mutations between codons 1403 and 1578 are associated with increased extracolonic manifestations such as desmoid tumors and mandibular lesions but patients with such mutations lack CHRPE (Davies et al., 1995). Likewise, colonic manifestations have been shown to vary with the position of the mutation. Truncating mutations amino terminal to codon 157 are associated with an attenuated form of FAP in which patients develop a relatively small number of polyps (Spirio et al., 1993). Some studies have suggested that mutations between codons 1250 and 1464 are associated with an increased

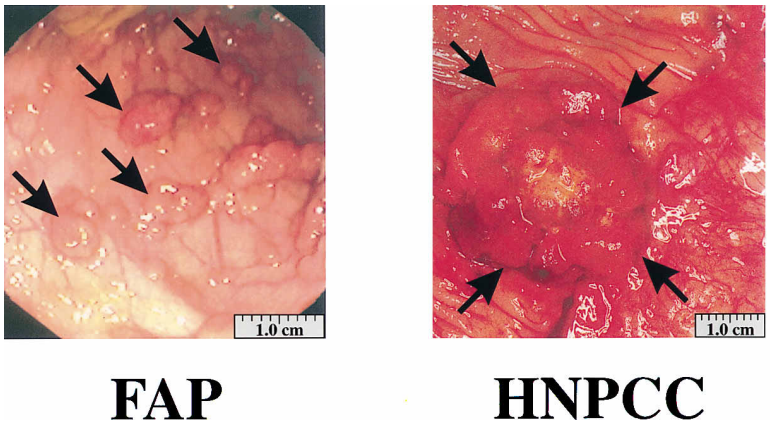

Figure 1. Examples of Colorectal Tumors Arising in FAP and HNPCC Patients

The left panel is a small portion of the colon from an FAP patient as viewed through the colonoscope, illustrating the multiple benign tumors (adenomas) characteristic of FAP (arrows). The right panel shows a single cancer from an HNPCC patient after surgical resection.

number of colorectal tumors (Nagase and Nakamura, 1993). On the other hand, patients with identical mutations can develop dissimilar clinical features. For example, some patients with identical truncating mutations develop features of Gardner's syndrome (mandibular osteomas and desmoid tumors) while others do not (Nishisho et al., 1991). Similarly, only a small number of patients within any kindred develop brain tumors, hepatoblastomas, or thyroid cancers, even though there is a clear predisposition to these tumors associated with germline *APC* mutations (Giardiello, 1995; Hamilton et al., 1995). The complex relationship between genotype and phenotype is also apparent in other hereditary cancer predisposition syndromes, including those associated with breast cancer (Szabo and King, 1995). Whether these phenotypic differences result from environmental influences or modifying genes is not known.

Perhaps the most clear-cut example of the distinction between phenotype and *APC* genotype is observed in MIN mice. These mice develop multiple intestinal adenomas and have a truncating mutation of the murine *APC* gene (*mAPC*) at a position similar to that found in many FAP patients (Su et al., 1992). Depending on the inbred mouse strain harboring this mutation, however, the number of polyps varies significantly. Linkage analysis has demonstrated that a single locus (*MOM1*, for modifier of MIN) on mouse chromosome 4 accounts for much of this difference between strains (Dietrich et al., 1993). Recently, the *MOM1* gene has been identified as that encoding secreted phospholipase A2 (sPLA2) (MacPhee et al., 1995).

Hereditary Versus Environment

The identification of *MOM1* as a phospholipase-encoding gene provides an excellent example of how genetic studies can lead to clues about the interaction between heredity and environment. As depicted in Figure 3, hereditary cancers clearly have a central genetic component, but there are important differences between them and classic genetic diseases such as cystic fibrosis and muscular dystrophy. In the latter cases, the genetic alterations lead to disease in a straightforward and reproducible fashion. Certain forms of diabetes and atherosclerosis present a more complex scenario, in which disease severity is significantly influenced by diet and metabolic interventions. Cancer is even more complex. Patients with germline mutations are predisposed to cancer, but will not necessarily be afflicted with disease. Additional mutations are required, and the rate of mutation can obviously be affected by environmental factors. In addition to affecting mutation rates, the diet may affect other cellular processes, like apoptosis, which could limit tumor initiation or progression (Bellamy et al., 1995).

Epidemiologic studies strongly suggest that the diet can influence colorectal cancer incidence (Giovannucci and Willett, 1994). However, human diets are so complex

Table 1. APC Mutations in Colorectal Neoplasia

	FAP	Sporadic Adenomas	Sporadic Cancers
Population incidence	1 in 7000	1 in 2	1 in 20
APC mutation prevalence	>85%[b] (Germline Mutations)	>80%[c] (Somatic Mutations)	>80%[d] (Somatic Mutations)
Nature of mutations[a]			
Truncating	96%[e]	89%[f]	98%[g]
Missense	4%[e]	11%[f]	2%[g]

[a] Based on APC mutations that could be precisely defined at the nucleotide level. For the purposes of this table, frameshift, nonsense, and splice site mutations were considered "truncating".
[b] Based on 62 kindreds (Powell et al., 1993).
[c] Based on analysis of 12 colorectal polyps (Jen et al., 1994).
[d] Based on analysis of 23 colorectal cancer cell lines (Smith et al., 1993).
[e] Based on 174 mutations (summarized in Nagase and Nakamura, 1993).
[f] Based on 19 mutations (Miyoshi et al., 1992; Powell et al., 1992).
[g] Based on 56 mutations (Miyoshi et al., 1992; Powell et al., 1992).

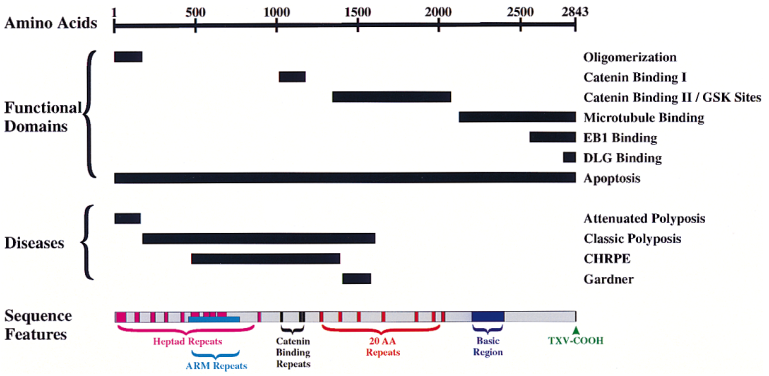

Figure 2. Functional and Pathogenic Properties of *APC*

Functional domains and sequence features: amino-terminal residues 1 to 171 are sufficient for oligomerization (Joslyn et al., 1993). This oligomerization is thought to be mediated by the heptad repeats indicated in pink on the sequence features map. APC binds to β-catenin through two motifs, the first comprising three 15 amino acid repeats indicated in black on the sequence features map and located between residues 1020 and 1169 (Rubinfeld et al., 1993; Su et al., 1993). A second region, comprising "20 aa repeats" (indicated in red on the sequence feature map and within residues 1324 to 2075; Groden et al., 1991) binds to β-catenin and also acts as substrate for GSK phosphorylation (Munemitsu et al., 1995; Rubinfeld et al., 1996). Phosphorylation is thought to occur at SXXXS sites within the 20 aa repeats (Rubinfeld et al., 1996). When transiently overexpressed, full-length APC decorates the microtubule cytoskeleton. The carboxyl terminus of APC is required for this association and residues 2130 to 2843 are sufficient (Munemitsu et al., 1994; Smith et al., 1994). Two proteins have been shown to associate with the carboxyl terminus of APC. Residues 2560 to 2843 are sufficient to bind EB1, a highly conserved 30 kDa protein of unknown function (Su et al., 1995). Residues 2771 to 2843 are sufficient to bind DLG, a human homolog of the Drosophila Disc large tumor suppressor gene (Matsumine et al., 1996); the three carboxy-terminal residue motif TXV (indicated in green on the sequence features map) probably mediates this binding. Expression of full-length APC in colorectal cancer cell lines results in apoptosis, but the regions required for this activity have not been precisely defined (Morin et al., 1996). Residues 453 to 767 contain 7 copies of a repeat consensus found in the Drosophila segment polarity gene product armadillo, as indicated in turquoise and residues 2200 to 2400 correspond to a basic region, indicated in blue (Groden et al., 1991).

Disease map: the location of truncating *APC* mutations has been shown to correlate with the extent of colonic and extra-colonic manifestations. Truncating mutations prior to codon 157 are associated with a reduced number of colorectal polyps (Spirio et al., 1993) whereas the majority of mutations are associated with more pronounced polyposis and occur between codon 169 and 1600 (Nagase and Nakamura, 1993). Mutation in codons 463 to 1387 are associated with congenital hypertrophy of the retinal pigment epithelium (CHRPE) (Olschwang et al., 1993). Mutations in codons 1403 to 1578 have been associated with Gardner's Syndrome, in which an increased incidence of extra-colonic manifestation is observed (Davies et al., 1995).

that it has been difficult to determine which dietary components are responsible for this modulation. The discovery of *MOM1* supports the idea that lipids are among the critical dietary components. The lipid content of diets varies dramatically, perhaps explaining geographic differences in colorectal cancer incidence and the higher rate of colorectal cancer associated with diets containing large amounts of red meat (Giovannucci and Willett, 1994). Moreover, it has been shown that nonsteroidal anti-inflammatory drugs (NSAID) such as sulindac can prevent tumor formation and even cause regression of existing colorectal tumors in both man and mouse (e.g., Giardiello et al., 1993; Boolbol et al., 1996). As sulindac inhibits the cyclooxygenases that metabolize the lipid arachidonic acid, it is possible that genetic studies of *MOM1*, epidemiologic studies correlating colorectal cancer incidence with diet, and chemopreventative studies with NSAID are all linked through lipids, particularly arachidonic acid.

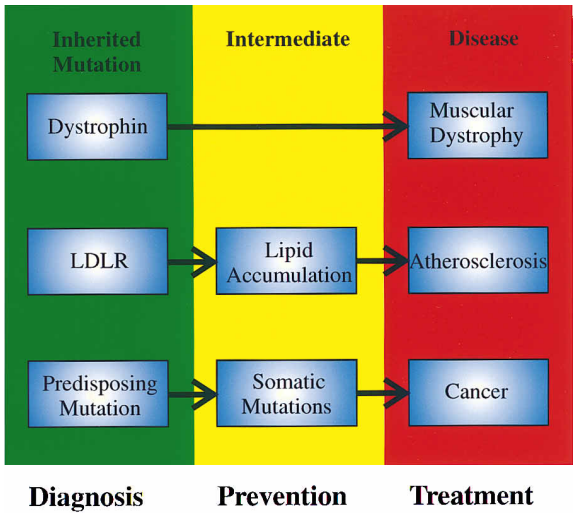

Figure 3. Comparison of Genetic Diseases

Three types of genetic diseases, of increasing complexity, are illustrated (see text).

Rare Syndromes, Common Cancers, and Gatekeepers

Studies of relatively rare inherited cancer predisposition syndromes like FAP have proved rewarding not only in their own right but also because the genes identified through such studies have been shown to play a role in the more common "sporadic" (i.e., nonfamilial) cancers of the same type (Table 1). For example, germline mutations of the *APC* gene cause FAP, a syndrome that accounts for <1% of colorectal cancers in the United States. But once *APC* was discovered, it was found that somatic mutations of *APC* occur in the great majority of sporadic colorectal tumors (Miyoshi et al., 1992; Powell et al., 1992). In the latter cases, *APC* mutations occur in single colonic epithelial cells, resulting in truncations of the APC protein identical to those observed in FAP patients.

Truncation of the *APC* gene may not be absolutely required for colorectal tumorigenesis, as ~15% of colorectal cancers apparently synthesize only full-length APC protein (Smith et al., 1993). Likewise, *APC* mutations do not appear to occur in all mutagen-induced rodent colorectal tumors (Kakiuchi et al., 1995). Whether

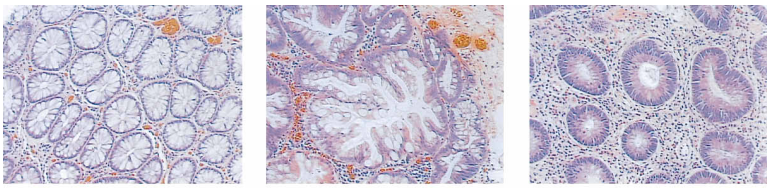

Figure 4. Histology of Normal and Neoplastic Colonic Epithelium

The left panel shows a group of normal colonic crypts in cross section. Note that the epithelial cells are precisely lined up along the basement membrane and that there is great uniformity among the glands. The center panel shows the morphology typical of a hyperplastic lesion. Individual cells are morphologically normal, but the increased number of cells in the crypts promotes crowding and mucosal folding, resulting in a saw-toothed appearance. The right panel shows morphology typical of a dysplastic ACF or adenoma. Note the increased nuclear/cytoplasmic ratio, the lack of uniform architecture, and the many nuclei that are no longer lined up along the basement membrane.

such tumors result from mutations of *APC* that are undetectable by standard methods or from mutations in other genes that encode regulators or mediators of APC function is an important area for future study.

How do *APC* mutations initiate colorectal tumorigenesis? Of the 100,000 genes in the human nucleus, why does mutation of one, and only one, gene lead to the development of polyposis? And why do patients with such inherited mutations not develop cancers in other organs, despite the fact that APC is ubiquitously expressed? We speculate that a single gene, *APC*, acts as the "gatekeeper" of colonic epithelial cell proliferation and that inactivation of this gatekeeper is required for net cellular proliferation. Normally, gatekeeper genes are responsible for maintaining a constant cell number in renewing cell populations, ensuring that cells respond appropriately to situations requiring net cell growth (e.g., tissue damage). A mutation of the gatekeeper leads to a permanent imbalance of cell division over cell death. Conversely, mutations of other genes in the presence of a normal gatekeeper would not lead to a sustainable growth perturbation.

Several observations are consistent with this hypothesis. Inactivation of both alleles of the murine *APC* gene occurs very early in mouse colon tumor development, as demonstrated by their occurrence in lesions so small that they can only be observed at the microscopic level (Levy et al., 1994; Luongo et al., 1994). In the human, too, complete *APC* inactivation has been found in the earliest neoplastic lesions that can be examined (Jen et al., 1994). Such lesions, called dysplastic aberrant crypt foci (ACF), are believed to be the precursors of adenomas.

What happens if mutations of other genes involved in colorectal cancer occur before those of *APC*? Apparently, such mutations do not efficiently initiate the neoplastic process. One example is provided by the tumor suppressor p53, which is genetically altered in >80% of colorectal cancers (Baker et al., 1990). Yet patients with germline mutations of p53 do not develop polyposis and in fact are not even at high risk to develop colorectal cancer (Garber et al., 1991). Therefore, though it is clear that p53 can play a role in colorectal tumorigenesis, it is equally clear that it cannot initiate the process in a fashion similar to *APC*.

It might be argued that the p53 protein is not expressed in normal colorectal epithelium and is presumably not involved in controlling the normal balance between colonic cell birth and death. Therefore, a mutation of p53 in an otherwise normal colonic epithelial cell may have no physiologic effect. In contrast, the RAS protein is expressed in normal colonic epithelium, and mutations of RAS frequently occur in colonic tumors as they progress (Vogelstein et al., 1988; Shibata et al., 1993). So what happens if a mutation of RAS occurs in a normal colonic epithelial cell? Interestingly, such mutations do not appear to lead to colorectal neoplasia (Jen et al., 1994). Cells with *RAS* gene mutations are amazingly common and form foci of hyperproliferating cells (Pretlow et al., 1993). But these cells have a normal intracellular and intercellular organization, unlike the dysplastic cells in the ACF-containing mutant *APC* genes (Figure 4) (Jen et al., 1994). Moreover, the hyperplastic cells containing mutant *RAS* genes, unlike their dysplastic counterparts with mutant *APC* genes, have little or no potential to form clinically important tumors and may eventually regress through apoptosis (Shpitz et al., 1996).

These studies suggest that it is not simply the accumulation of mutations, but rather it is also their order, that determines the propensity for neoplasia, and that only a subset of the genes which can affect cell growth can actually initiate the neoplastic process. Though APC is expressed ubiquitously, it may function as the gatekeeper only in colorectal epithelium. In other cell types, its function may be redundant or at least expendable, and different gene products presumably perform the gatekeeper role. Other potential gate-keepers include the *NF1* gene in Schwann cells, the *Rb* gene in retinal epithelial cells, and the *VHL* gene in kidney cells (reviewed in Knudson, 1993). Unfortunately, no gatekeeper has yet been discovered for the cell types accounting for the great majority of human malignancies, including those of the lung, breast, prostate, pancreas, brain, and bladder. Progress in understanding the pathogenesis of these malignancies will in large part depend on identifying these gatekeepers.

Gatekeeper Mechanisms

How does *APC* exerts its gatekeeper effect? The APC protein is apparently located at the basolateral membrane in colorectal epithelial cells, with expression more pronounced as cells migrate up through the crypt column (Smith et al., 1993; Miyashiro et al., 1995). Expression of wild-type APC in colorectal epithelial cells with *APC* mutations results in apoptosis, suggesting that APC may control the cell death process (Morin et al., 1996). Abrogation of such a "death signal" could clearly alter the precise homeostatic balance required in renewing cell populations.

The protein encoded by APC consists of 2843 amino acids without strong similarities to proteins of known function (Figure 2). The amino-terminal third of APC contains several heptad repeats of the type that mediate oligomerization by a coiled-coil structure (Joslyn et al., 1993). These regions may mediate homo-oligomerization between mutant and wild-type proteins, and could theoretically cause a dominant negative effect, though no such effect has been demonstrated biologically.

While the primary sequence of *APC* has provided few insights into its function, the identification of proteins that interact with APC has yielded tantalizing clues. Two proteins that bind to the C-terminus of APC have been identified. One of these is EB-1, a highly conserved 30 kDa protein of unknown function (Su et al., 1995). More recently, the carboxy-terminal 72 residues of APC were demonstrated to bind the human homolog of the Drosophila tumor suppressor gene discs large (*DLG*) (Matsumine et al., 1996). As virtually all *APC* mutations result in the loss of the carboxyl terminus of APC protein (Table 1A), these data suggest that DLG and/or EB1 may be essential for APC's growth-controlling function. However, the most telling insights to APC function come from studies of the interaction between β-catenin and APC (Rubinfeld et al., 1993; Su et al., 1993). The central third of APC harbors two classes of β-catenin binding repeats, one of which is modulated by phosphorylation (Figure 2) (Rubinfeld et al., 1996). Although many mutant APC proteins retain some β-catenin binding, virtually all mutant APC proteins lack at least one type of β-catenin binding repeat.

The β-catenin association links APC to two apparently disparate cellular processes. The first process is related to cellular adhesion. The catenins were originally identified as cytoplasmic proteins that bind to cadherins, a family of calcium-dependent homophilic cell adhesion molecules. Several studies have indicated that β-catenin is necessary for cadherin-mediated cell adhesion (reviewed in Kemler, 1993). Given that binding of β-catenin to cadherins or to APC is mutually exclusive, it is possible that APC could modulate such adhesion as part of its gatekeeping function. APC could additionally act as a downstream communicator of adhesion status, linking cadherin-catenin complexes to other cellular components.

The second process involving β-catenin has been elucidated via studies of the Wingless (Wg) and Wnt signaling pathways in Drosophila and mouse, respectively (Figure 5). β-catenin and Armadillo (the Drosophila homolog of β-catenin) have been firmly implicated as signal transducers in these pathways (Gumbiner, 1995). This link was strengthened by the observation that the APC/β-catenin complex is physically associated with a second member of this pathway, the ZW3/GSK3β protein kinase (Rubinfeld et al., 1996). This kinase was found to promote β-catenin binding to APC, presumably by phosphorylation of APC Class II binding sites (Figure 2). Epistasis evaluations in Drosophila suggest that Wg signaling inhibits ZW3 function, and that active ZW3 can inhibit β-catenin signaling. Finally, recent studies in Xenopus and mouse suggest that Wnt signaling ultimately results in formation of a heteromeric complex containing β-catenin and members of the Tcf/Lef family

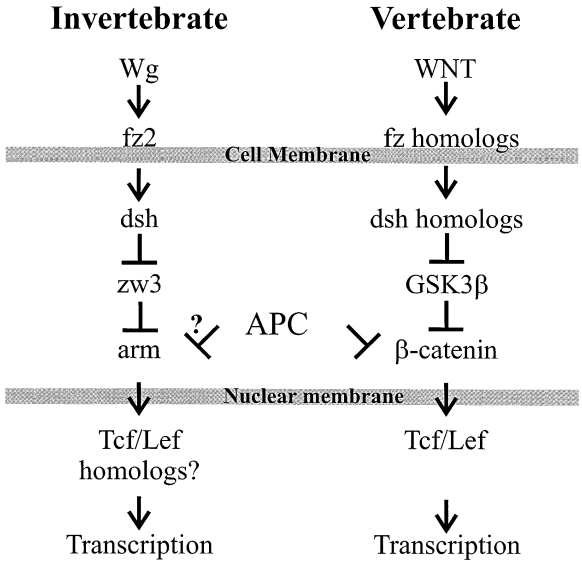

Figure 5. β-Catenin in the Wg and WNT Signaling Pathways

The Drosophila and mouse Wg/WNT signaling pathways are diagrammed as summarized in Gumbiner, 1995, and extended by addition of the Wg receptor fr2 (Bhanot et al., 1996) and the catenin associated transcription factors Tcf-3 and Lef-1 (Behrens et al., 1996; Molenaar et al., 1996). The biochemical attributes of the protein encoded by disheveled (dsh) is not known, and a Drosophila homolog of *APC* has not yet been described. See text for further details.

of HMG box transcription factors (Behrens et al., 1996; Molenaar et al., 1996). Taken together, the studies suggest that APC could work in concert with ZW3/GSK3β to inhibit β-catenin-induced transcriptional activity. The importance of this pathway in neoplasia is supported by the ability of truncated β-catenin to transform cells in culture (Whitehead et al., 1995) and the involvement of Wnt signaling in breast tumorigenesis in mice (Nusse and Varmus, 1992). Given the size of APC, the biological manifestations of APC inactivation and the number of proteins already known to associate with APC, it is likely that APC functions to integrate signals from a variety of sources, transmitting them to the nucleus through β-catenin/Tcf complexes.

One general lesson from both the armadillo/β-catenin and DLG work is that basic research on the development of invertebrates can illuminate pathways involved in human tumorigenesis. If the complex signaling pathways mediating tumor suppressor gene functions are ever to be understood, it is likely that the understanding will be heavily indebted to the powerful experimental systems available in such eukaryotes. The studies of HNPCC described below provide another example of how such basic research in nonhuman systems has proved pivotal for unraveling the mechanisms underlying colorectal neoplasia.

Hereditary Nonpolyposis Colorectal Cancer

HNPCC exemplifies the problems inherent in identifying the genetic components of diseases that are common in the population. Similar challenges have been posed by breast cancer, melanomas, diabetes, and a variety of

Table 2. MMR Genes in Colorectal Neoplasia

	HNPCC	Sporadic Adenomas	Sporadic Cancers
Population incidence	~1 in 500	1 in 2	1 in 20
MMR deficiency prevalence[a]	>90% of kindreds[c]	<3%[d]	13%[e]
MMR gene mutations	>70%[f]	UNKNOWN	~65% of CRC with MI[g]
hMSH2	45%[f]	—	60%[h]
hMLH1	49%[f]	—	35%[h]
hPMS2	6%[f]	—	5%[h]
Nature of Mutations[b]			
Truncating	70%[i]	—	55%[h]
Missense	30%[i]	—	45%[h]

[a] As assessed by presence of microsatellite instability (MI).
[b] Based on MMR mutations that could be precisely defined at the nucleotide level. For the purposes of this table, frameshift, nonsense, and splice site mutations as well as large intragenic deletions were considered "truncating." Three basepair deletions were counted as missense mutations.
[c] Based on analysis of colorectal tumors from 74 kindreds (Liu et al., 1996).
[d] Based on analysis of 46 adenomas (Young et al., 1993).
[e] Based on analysis of 273 colorectal cancers (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993).
[f] Based on 33 mutations in 47 kindreds. In addition, a *hPMS1* mutation was identified in a single kindred (Liu et al., 1996).
[g] Based on 15 cases published as of September 1, 1996.
[h] Based on 20 somatic mutations published as of September 1, 1996.
[i] Based on 89 germline mutations published as of September 1, 1996.

psychiatric conditions. The confounding factors include chance clusterings mimicking familial forms of disease, the influences of environmental factors (such as diet) on penetrance, genetic heterogeneity, and phenocopies (individuals within afflicted families with the same disease but not sharing the causative mutation). In fact, though HNPCC was suspected to be heritable over 80 years ago, it remained obscure and understudied as a result of these problems. It was only through the sustained efforts of clinical epidemiologists in the last 20 years that HNPCC finally became recognized as a bonafide hereditary disease (Lynch et al., 1996).

Within the last three years, substantial advances in understanding the molecular pathogenesis of HNPCC has been made as a result of three related lines of investigation. The first was linkage analysis. Following exclusion of candidate gene loci, large kindreds from North America, Europe, and New Zealand were evaluated using microsatellite markers distributed throughout the genome. Tight linkage to either chromosome 2p16 or 3p21 was identified in individual families, providing unambiguous evidence that HNPCC was a simple Mendelian disease (Lindblom et al., 1993; Peltomaki et al., 1993).

The second clue was found during attempts to demonstrate allelic losses with the 2p16 microsatellite markers linked to HNPCC susceptibility (Aaltonen et al., 1993). Such losses are often found associated with tumor suppressor loci. In HNPCC tumors, however, new microsatellite alleles, not found in the patient's normal cells, were observed instead of the expected allelic losses. These new alleles were evident in every dinucleotide and trinucleotide repeat examined, suggesting a genome-wide instability of the replication or repair of simple repeated sequences. This form of instability was similar to that first described in a subset of sporadic (nonfamilial) colorectal cancers (Peinado et al., 1992; Ionov et al., 1993; Thibodeau et al., 1993). Similarities between certain biologic features of the sporadic and hereditary tumors with microsatellite instability (MI) suggested that related mechanisms might be involved (Ionov et al., 1993).

The third clue was provided not by tumor biologists, but by investigators studying replication fidelity in unicellular organisms (Strand et al., 1993). These investigators recognized that the microsatellite instability observed in tumors was similar to that observed in bacteria harboring mutations in mismatch repair (MMR) genes such as *mutS* and *mutL*. Analogous experiments in yeast showed that the microsatellites observed to be unstable in HNPCC patients were equally unstable in yeast with defective MMR genes, and it was specifically hypothesized that HNPCC was caused by hereditary mutations of human homologs of *mutS* or *mutL* (Strand et al., 1993). These insights stimulated a search for such human homologs, resulting in the discovery of five human genes likely to participate in MMR in addition to one serendipitously discovered in 1989 (reviewed in Marra and Boland, 1995). That a *mutS* homolog might be involved in the form of HNPCC linked to chromosome 2 was suggested by the finding that one such homolog (*hMSH2*) was located on chromosome 2p (Fishel et al., 1993). Direct evidence that an MMR gene was involved in the disease was provided by the identification of germline mutations of *hMSH2* in HNPCC kindreds (Leach et al., 1993). This was soon followed by the identification of mutations of *mutL* homologs in other HNPCC kindreds. It is currently believed that mutations in three human MMR genes (*hMSH2*, *hMLH1*, and *hPMS2*) account for the great majority of HNPCC kindreds (Table 2).

Strong supporting evidence that *mutS* and *mutL* genes played a role in HNPCC was provided by biochemical experiments (Parsons et al., 1993; Umar et al., 1994). Extracts of tumors with MI were found to be completely deficient in mismatch repair activity in vitro. The nature of the biochemical defect suggested that one of the initial stages of the MMR process, which involve *mutS* and *mutL* in bacteria, was defective (Figure 6). Finally, transfer of a human chromosome containing the normal copy of *hMLH1* into a human cancer cell line with a mutant *hMLH1* gene completely restored MMR activity and reversed the MI (Koi et al., 1994).

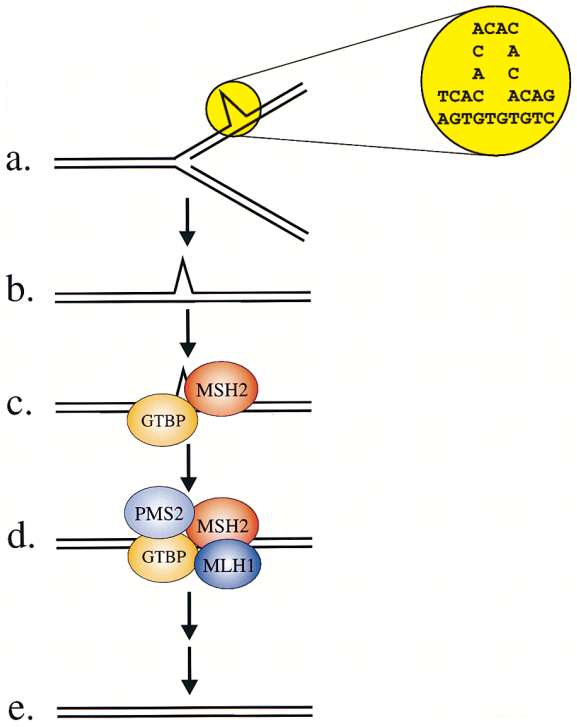

Figure 6. MMR Gene Pathway in Human Cells

(a and b) During the normal course of DNA replication, single base mismatches may result from misincorporation by polymerases (not shown) and larger mismatches may result from strand slippage (illustrated in yellow).

(c) The resulting mismatch is recognized by mutS homologs. In humans, optimal mismatch recognition is thought to require at least two mutS homologs, MSH2 and GTBP. Another *mutS* homolog, *MSH3*, may substitute for GTBP in certain cases (Palombo et al., 1996).

(d and e) MutL homologs are then recruited (d) to the complex and (e) the mismatch is repaired by a process that in bacteria involves an exonuclease, helicase II, DNA polymerase III, single-stranded binding protein, and DNA ligase (Modrich, 1995).

Genetic Instability in Cancer

The idea that multiple mutations are necessary for the development of malignancy is now widely accepted and well illustrated by studies of colorectal cancer (Figure 7). As both alleles of each of the tumor suppressor genes indicated in the figure are inactivated during this process, at least seven independent genetic events appear to be required. Are normal rates of mutation sufficiently high to account for these accumulated mutations, or do tumor cells have intrinsically high rates of mutation? This question has been debated for years without resolution (Loeb, 1991). The studies on HNPCC have provided a definitive answer to this question at least in a subset of cancers by demonstrating that mutation rates in tumor cells with MMR deficiency are two to three orders of magnitude higher than in normal cells (Bhattacharyya et al., 1994; Shibata et al., 1994; Eshleman et al., 1995).

HNPCC accounts for 2%–4% of the total colorectal cancers in the Western world (Lynch et al., 1996). Sporadic tumors with microsatellite instability account for another 13% of the total colorectal cancers (Table 2), but the molecular mechanisms underlying instability in the sporadic cases are incompletely defined. Some of them result from somatic mutations of the same MMR genes causing HNPCC, while others have mutations of *GTBP* or *MSH3*, mismatch repair genes rarely mutated in HNPCC patients (Papadopoulos et al., 1995; Malkhosyan et al., 1996) or in the proofreading domain of polymerase δ (da Costa et al., 1995). In other colorectal tumors with MI, no mutations of repair genes have been identified (Liu et al., 1995).

The 85% of colorectal cancers that do not exhibit MI also do not exhibit high rates of mutation in standard assays (Bhattacharyya et al., 1994; Eshleman et al., 1995). However, each of these other tumors does in fact have a large number of genetic alterations, characterized by losses of large chunks of chromosomes. These losses result from mitotic recombination or aberrant mitotic segregation of chromosomes. At the molecular level, colorectal cancers without MI lose an average of at least 25% of randomly chosen alleles, while cancers with MI often lose none (Aaltonen et al., 1993). At the cytogenetic level, these changes are reflected by aneuploidy in the former cancers and euploidy in the cases with MI (Bocker et al., 1996). Teleologically, it would thus seem that a cancer needs to develop only one type of instability and that gross chromosomal changes provide little selective growth advantage to tumors with mismatch repair deficiency and vice versa. It would also seem that there are at least two ways for a tumor to develop the multiple genetic alterations required for malignancy: subtle alterations due to the mismatch repair deficiency occur in a minority of cases (those with MI), while gross chromosomal alterations occur in the majority.

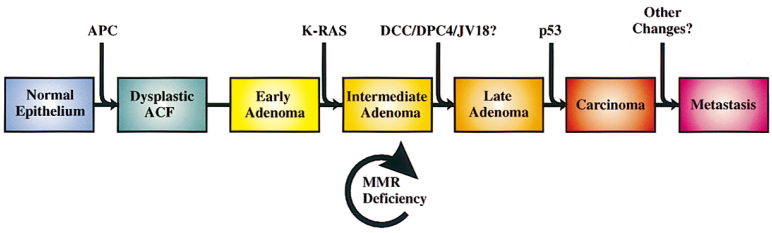

Figure 7. Genetic Changes Associated with Colorectal Tumorigenesis

APC mutations initiate the neoplastic process, and tumor progression results from mutations in the other genes indicated. Patients with FAP inherit APC mutations and develop numerous dysplastic aberrant crypt foci (ACF), some of which progress as they acquire the other mutations indicated in the figure. The tumors from patients with HNPCC go through a similar (Huang et al., 1996), though not identical (Markowitz et al., 1995), series of mutations; MMR deficiency speeds up this process. K-RAS is an oncogene that requires only one genetic event for its activation. The other specific genes indicated are tumor suppressor genes that require two genetic events (one in each allele) for their inactivation. Chromosome 18q21 may contain several different tumor suppressor genes involved in colorectal neoplasia, with *DCC*, *DPC4*, and *JV18–1* genes proposed as candidates. A variety of other genetic alterations have each been described in a small fraction of advanced colorectal cancers. These may be responsible for the heterogeneity of biologic and clinical properties observed among different cases.

An important implication of these studies relates to the process of aneuploidy in general. Aneuploidy has long been observed to be a hallmark of cancer cells. But does aneuploidy simply reflect the numerous extra cell divisions, abnormal microenvironment, and altered physical structure of the cancer cell, or does it represent a specific physiologic defect that is causally involved in tumorigenesis? Such distinctions between cause and effect in properties associated with cancer are extremely difficult to resolve. The HNPCC work supports the hypothesis that aneuploidy is causally related to neoplasia. If aneuploidy were simply the consequence of the neoplastic factors described above, it should be found in colorectal tumors with MMR deficiency as often as in other colorectal tumors.

Apart from differences in the type of genetic instability in tumors with MI, other important distinguishing features of these tumors have been described. MI-containing tumors have defects in HLA-locus genes, perhaps allowing them to evade the immune response that might be expected to be generated by tumors with multiple, random mutations (Branch et al., 1995). Whether colorectal tumors without MI have similar alterations in proteins participating in immune recognition is not known. In addition, colorectal tumors are generally insensitive to the growth-suppressing hormone TGFβ. In tumors with MI, this insensitivity is almost always due to frameshift mutations within a microsatellite sequence embedded in the TGFβ receptor II gene (Markowitz et al., 1995). In tumors without MI, mutations of this receptor have not been detected. Instead, the TGFβ insensitivity in some of these cases probably results from mutations of human MAD homologs that transmit growth-inhibitory signals from the TGFβ receptors (Hahn et al., 1996; Riggins et al., 1996).

A small fraction of many tumor types, in addition to those of the colon, display some level of MI (summarized in Dams et al., 1995). In most of these tumors, the instability is considerably less pronounced than that observed in colon tumors and is unlikely to be due to mismatch repair gene defects. A large fraction of these other types of tumors exhibit gross chromosomal changes, but as in the colon, the mechanisms underlying such abnormalities are unknown, representing a major challenge for the future.

Mutagens and Cancer

The normal tissues from HNPCC patients do not generally display genetic instability or a biochemical defect in MMR. However, a few HNPCC patients have been identified that display elevated rates of mutations in their phenotypically normal cells, accompanied by a biochemically detectable deficit in MMR (Parsons et al., 1995). This defect may be due to inheritance of a dominant negative mutation, whereas most MMR gene mutations are null. The patients with such mutations are remarkably normal. In particular, while these patients develop colorectal cancers at an early age, similar to that of other HNPCC patients, they do not display the exponential increase in cancer incidence that would be predicted if acquisition of several somatic mutations was the only rate-limiting step required for cancer development. Similarly, mice with targeted disruptions of MMR genes and devoid of MMR activity develop surprisingly few tumors (e.g., Reitmair et al., 1996).

If humans or mice were continually exposed to mutagens at doses sufficient to induce the grossly elevated rate of mutation observed in MMR-deficient cells, one would have expected a great number of tumors of many different types. Why isn't MMR deficiency as carcinogenic as mutagen exposure? One possibility is that mutagens are carcinogenic not only because they induce mutations, but also because they cause substantial cellular death with consequent tissue regeneration (Ames and Gold, 1990; Cohen and Ellwein, 1990). We hypothesize that in cells in which mutations of growth controlling genes occur, apoptosis results and prevents such cells from proliferating. Apoptosis has been shown to be a potent mechanism for killing cells receiving conflicting growth stimulatory and inhibitory signals (Bellamy et al., 1995). In MMR-deficient cells, the apoptosis would serve as a safeguard to prevent neoplasia. In regenerating tissues, however, the apoptotic signals must be turned off or the net cellular growth required for regeneration would be impossible. Mutations in growth-controlling genes in this environment would therefore lead to the initiation or progression of neoplasia, while in uninjured cells, the same mutations would simply result in cell death.

This argument is speculative but may help to explain some puzzling observations relating diet to colorectal cancer incidence. There is little question about the importance of diet in limiting colorectal cancer incidence in the Western world. It has been a reasonable assumption that the dietary components responsible were mutagens. However, examination of mutational spectra in colorectal cancers has provided little evidence to favor specific mutagens as causative agents. The most characteristic mutations observed in p53 and *APC* genes, for example, are C-to-T transitions at CG dinucleotides (Harris and Hollstein, 1993). Such mutations are characteristic of endogenous processes leading to the hydrolytic deamination of methylated C residues in the absence of mutagen exposure. In contrast, the mutational spectra in cancers of the skin, liver, and lung are characteristic of DNA damage associated with specific environmental components (Harris and Hollstein, 1993). Thus, it is possible that the dietary factors which lead to colorectal cancer are not mutagens, but rather irritants that lead to tissue regeneration. Dietary fibers may absorb these irritants, explaining part of their protective effect (Giovannucci and Willett, 1994). This hypothesis is also consistent with the observation that the age of incidence of colorectal cancer appears to be decreasing in HNPCC kindreds. The mutation rate in such MMR-deficient cancers is already extremely high, and it is unlikely that dietary mutagens could increase this rate significantly. However, these diets may have become progressively more irritating to the bowel due to decreases in fiber and increases in other constituents over the course of the last century.

FAP Versus HNPCC

Tumor evolution in the two inherited colorectal cancer syndromes described above provide an illuminating

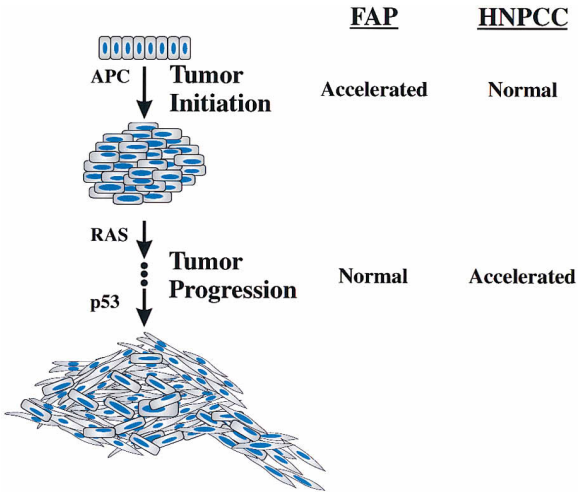

Figure 8. Comparison of the Development of Cancer in FAP and HNPCC Patients

FAP results from an increased rate of tumor initiation due to abrogation of the gatekeeper function of APC. In contrast, the mismatch repair defect in HNPCC results in an enhanced rate of mutation that greatly accelerates tumor progression but results in near normal rates of tumor initiation. That the rate of tumor initiation is nearly normal in HNPCC patients is indicated by the fact that such patients do not have a greatly increased incidence of adenomas, in striking contrast to FAP patients (Lynch et al., 1996). But FAP and HNPCC patients both develop colorectal cancers at a median age of 42 years, suggesting that tumor progression is as rate-limiting for cancer formation as is tumor initiation.

contrast (Figure 8). FAP is a disease caused by a faulty gatekeeper, *APC*. This defect allows thousands of benign tumors to form, but each of these tumors slowly progresses to malignancy, requiring the sequential accumulation of mutations in *RAS*, *p53*, etc. Because there are so many adenomas, however, at least some will progress to cancer. The median age of cancer diagnosis in untreated FAP patients is 42, 25 years earlier than the median age of sporadic colorectal cancer patients. In HNPCC, adenomas form at approximately the same rate as in the general population. However, the adenoma cell with the MMR deficiency acquires mutations at a rate two to three orders of magnitude higher than in normal cells, and the resultant accumulation of mutations in oncogenes and tumor suppressor genes leads to a rapid progression to malignancy. This accelerated rate of tumor progression is observed in mice with germline mutations of the murine *MSH2* gene as well as in humans with HNPCC (Lynch et al., 1996; Reitmair et al., 1996).

Interestingly, cancer in HNPCC patients occurs at a median age of 42, the same age as in FAP patients. FAP can therefore be considered as a disease of tumor initiation and HNPCC one of tumor progression. Initiation and progression are of course the cardinal features of malignancy. Many carcinogenesis studies have tended to focus on agents that initiate tumorigenesis. The studies on HNPCC suggest that agents which accelerate progression can be at least as important.

Clinical Implications

Knowledge of the hereditary bases of familial colorectal cancer has important implications for patients. The most immediate of these is improved diagnosis through genetic testing. Though the genes involved are known, development of practical methods for detecting germline alterations is not a simple matter. The genes are very large (*APC*) or multiple (MMR), and in both FAP and HNPCC the mutations are heterogenous.

In FAP, the development of specific tests has been facilitated by the knowledge that virtually all mutations so far described result in truncations of the APC protein through point mutations creating nonsense codons or small insertions or deletions causing frameshifts (Table 1A). Simple tests to detect such truncating mutations have been devised (Powell et al., 1993; van der Luijt et al., 1994). These involve the amplification of the *APC* transcript by RT–PCR and transcription and translation of the PCR product in vitro. Electrophoresis of the products of the reactions directly reveals truncated products when mutations exist. This assay detects mutations in ~85% of FAP kindreds. Similar tests reveal mutations in 50%-60% of HNPCC kindreds (Liu et al., 1996).

The remaining FAP and HNPCC kindreds present unsolved problems in diagnosis. Additionally, many individuals have a family history of colorectal cancer that does not meet the stringent criteria used to clinically define FAP and HNPCC. Most of these patients do not have germline mutations of either *APC* or MMR genes, but many are likely to wish to be tested for such mutations. Combined with the insensitivity of present assays, this suggests that only a small fraction of patients who might be tested for germline mutations will actually benefit from the test. In the remainder, the negative test results cannot be interpreted unambiguously, as they could represent either the absence of mutation or the insensitivity of the assay. These arguments underscore the need to develop more sensitive and cost-effective tests for genetic diagnosis. They also underscore the necessity of performing such tests under the supervision of trained genetic counselors who can accurately communicate the implications of positive or negative tests in a socially, medically, and psychologically sound fashion.

What are the ethical implications of such genetic testing? After all, neither HNPCC nor FAP is necessarily lethal. Appropriate surveillance and surgical intervention allows most patients to achieve normal life-spans, though with significant morbidity. Should prenatal testing for such nonlethal disease be permitted? Should information on genetic susceptibility be communicated to employers, insurance companies, other family members, prospective spouses? These questions are difficult and not limited to hereditary colorectal cancer—the identical issues are or will soon be faced with regards to breast cancers, melanomas, and a variety of other common diseases with genetic components.

Despite these problems, the positive attributes of genetic testing for cancer susceptibility should not be underestimated. Family members who have tested negative for the particular APC or MMR gene mutation involved in their kindred will be spared the repeated medical and endoscopic examinations that would otherwise have to be done on every member of each family. At least as importantly, such children and their parents are spared the considerable anxiety associated with not knowing whether they are affected. Surveillance can then be concentrated on those who have inherited a

mutant gene. Periodic colonoscopies in HNPCC patients should allow detection of tumors at early stages, when they can be removed by simple surgical procedures. In FAP patients, colectomies must now be performed to prevent colorectal cancer because there are so many benign tumors at risk for progression to malignancy. However, in the future, chemopreventative agents may inhibit the development of adenomas if they can be administered prior to or at the onset of neoplasia. Indeed, as noted above, NSAID can shrink existing adenomas in FAP patients and inhibit adenoma formation in MIN mice.

Acknowledgments

The authors are grateful to the members of their laboratories for their contributions to the reviewed studies and for their critical reading of the manuscript and to F. Giardiello and S. Hamilton for photographs of colorectal lesions. The authors are supported by Public Health Service grants CA 43460, CA 57345, and CA 62924. B. V. is an Investigator of the Howard Hughes Medical Institute.

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