Genetic Alterations in the Adenoma–Carcinoma Sequence

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Tumorigenesis is thought to be a multistep process in which genetic alterations accumulate, ultimately producing the neoplastic phenotype. A model was proposed to explain the genetic basis of colorectal neoplasia that included several salient features. First, colorectal tumors appear to occur as a result of the mutational activation of oncogenes coupled with the inactivation of tumor-suppressor genes. Second, mutations in at least four or five genes are required to produce a malignant tumor. Third, although the genetic alterations often occur in a preferred sequence, the total accumulation of changes, rather than their chronologic order of appearance, is responsible for determining the tumor's biologic properties. Several different genetic alterations were identified that occur during colorectal tumorigenesis. Activational mutation of the *ras* oncogene was found in approximately 50% of colonic carcinomas and in a similar percentage of intermediate-stage and late-stage adenomas. Allelic deletions were discovered of specific portions of chromosomes 5, 17, and 18, which presumably harbor tumor-suppressor genes. The target of allelic loss events on chromosome 17 has been shown to be the *p53* gene, which is mutated, not only in colonic cancer, but also in a large percentage of other human solid tumors. The gene *dcc* recently was identified; this candidate tumor-suppressor gene on chromosome 18 appears to be altered in colorectal carcinomas. The protein encoded by the *dcc* gene has significant sequence similarity to neural cell adhesion molecules and other related cell-surface glycoproteins. By mediating cell–cell and cell–substrate interactions, this class of molecules may have important functions in mediating cell growth and differentiation. Alterations of the *dcc* gene may interfere with maintenance of these controls and thus may play a role in the pathogenesis of colorectal neoplasia. Another candidate tumor-suppressor gene also was identified on chromosome 5, mcc (for mutated in colorectal cancers). The *mcc* genetic alterations include one tumor with somatic rearrangement of one *mcc* allele and several tumors with somatically acquired point mutations in the coding region. Studies currently are ongoing to (1) identify additional tumor-suppressor gene candidates, (2) increase our understanding of normal tumor-suppressor gene function, and (3) demonstrate the functional tumor-suppressor ability of these genes both in vivo and in vitro. *Cancer* 1992; 70:1727–1731.

Tumorigenesis is thought to be a multistep process in which genetic alterations accumulate, ultimately producing the neoplastic phenotype. The hypothesis that genetic damage might be responsible for cancer is not new; hereditary predispositions to cancer, damaged chromosomes in cancer cells, and DNA damage mediated by chemical carcinogens have been recognized for a long time. Genetic alterations in tumorigenesis vary and include amplification, point mutation, rearrangement, and deletion of specific genes. We recently proposed a model for the genetic basis of colorectal neoplasia with the following salient features.[1] First, colorectal tumors appear to occur as a result of mutational activation of oncogenes coupled with the inactivation of tumor-suppressor genes. Second, mutations in at least four or five genes are required for malignant transformation. Third, although the genetic alterations often occur according to a preferred sequence, the total accumulation of changes, rather than their chronologic order of appearance, is responsible for determining the tumor's biologic properties.

Epithelial tumors of the colorectum provide an excellent system in which to study genetic alterations and the manner in which they affect tumor progression. Colorectal tumors progress through a series of easily recognizable clinicopathologic stages. Tumorigenesis may be preceded by widespread hyperproliferation of colonic

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epithelial cells, which can be detected by in situ DNA labeling techniques.[2] One or a few of these hyperproliferating cells may undergo clonal expansion[3] and form a small benign neoplasm called a tubular adenoma. Progression of the adenoma is marked by a gradual increase in tumor size, acquisition of cytologic atypia, and development of villous architecture. After an adenoma cell develops the capacity to overgrow its sister cells and invade the basement membrane, the tumor is, by definition, a carcinoma, which eventually may acquire the ability to metastasize to regional lymph nodes and distant sites. This tumor progression is gradual and usually slow, requiring decades for culmination.

Several genetic changes accompany tumor progression and are, at least in part, responsible for it. One of the earliest recognizable changes is a significant loss of methyl groups in DNA.[4] Using restriction-endonuclease analysis, substantial hypomethylation was found in both adenomas and carcinomas compared with normal mucosa. The role of this hypomethylation in tumorigenesis is unclear, but we believe that hypomethylated DNA may inhibit chromosome condensation during mitosis and that these undercondensed regions may adhere to one another during anaphase, leading to aneuploidy.

Mutations in the *ras* gene are found in approximately 50% of colorectal carcinomas[5,6] and in a similar percentage of intermediate-stage and late-stage adenomas.[7] Most of these mutations are in the K-*ras* gene. In several tumors studied, the same mutation was found in both the adenomatous and carcinomatous portions of the same clinical lesion, suggesting that the mutation preceded the malignant transformation. By contrast, *ras* gene mutations are present in less than 10% of small (< 1 cm) adenomas. Although the full effects of mutated *ras* genes currently are not known, such genes can transform immortalized cell lines and cooperate with other oncogenes to transform primary cells in culture.[8-10] Some of the same *ras* mutations have been identified in the *ras* genes of RNA tumor viruses and in the cellular *ras* homologues of animal tumors induced by carcinogens.[11,12] Mutations in the *ras* gene occur at similar frequencies in the colorectal tumors of patients with and without familial adenomatous polyposis, suggesting that some of the molecular genetic events responsible for tumor progression are identical in both groups of patients.

Allelic loss of specific chromosomal regions, presumably harboring tumor-suppressor genes, are another type of genetic alteration identified in colorectal neoplasia. Using polymorphic DNA probes, the extent and nature of allelic loss was evaluated in 56 pairs of colorectal carcinomas and adjacent normal mucosa.[13] Termed "allelotyping," this analysis showed that allelic deletions were common and that eight chromosomal regions were each affected in more than 25% of the carcinomas studied. A median of four to six allelic losses was observed in individual carcinomas. Some of these losses may be "passengers," coincidentally occurring in the same mitosis as another genetic alteration that provided a selective growth advantage. However, we believe that many of these changes reflect the presence of tumor-suppressor genes in the deleted regions. Another model of tumorigenesis was proposed in which defective genes involved in inherited neoplastic syndromes normally function as tumor-suppressor genes.[14] At the cellular level, these genes act in a recessive manner (i.e., both alleles must be inactivated to promote tumor growth). The recent cloning and characterization of the retinoblastoma susceptibility *rb* gene supports this hypothesis.[15,16]

Deletions on the short arm of chromosome 17 (17p) are common and occur in more than 75% of colorectal carcinomas. These deletions appear to be relatively late events in colorectal tumor progression and are associated with the transition from the benign adenoma to malignant carcinoma. Using 20 polymorphic chromosome 17p markers, the common region of deletion in the carcinomas was localized to a region centered at 17p13.[17] The *p53* gene that encodes the transformation-associated protein is contained in this region.[18] Originally, *p53* was believed to be an oncogene, based on its ability to immortalize primary rodent cells and cooperate with *ras* during transformation.[19-22] Recent evidence has shown, however, that the *p53* derivatives used in these experiments were mutants and that normal *p53* may function as a tumor-suppressor gene rather than an oncogene.[23,24] We initially examined the *p53* coding region of two colorectal carcinomas in detail. Both tumors had lost one *p53* allele and expressed considerable messenger RNA from the remaining allele. Mutations in highly conserved regions of the *p53* gene were found in both tumors.[17] Subsequent studies have shown that *p53* genetic mutations are found routinely in colorectal carcinomas.[25,26] Furthermore, in a carcinoma with two retained parental 17p alleles, one allele contained a point mutation and both the wild-type and mutant alleles were expressed at equal levels.[25] Such findings led us to speculate that a mutated *p53* gene in a colorectal carcinoma can provide a selective growth advantage by promoting tumor progression even in the presence of a normal *p53* allele. Deletion of the remaining allele amplifies the growth advantage and leads to additional progression, often manifested as the transition from the

benign to the malignant state. Mutations in *p53* have been identified in many other types of human solid tumors, including those of the brain, breast, lung, bladder, and soft tissue.[25,27–29] Recently, germline *p53* genetic mutations were identified in DNA from patients with Li–Fraumeni syndrome, a rare disorder in which affected patients are at high risk for several different types of malignant lesions at early age.[30]

Allelic losses on chromosome 18q also are common. They occur in approximately 75% of colorectal carcinomas. Deletions can be seen in 47% of large adenomas and in less than 10% of small and intermediate-stage adenomas. This suggests that 18q loss events generally occur before *p53* genetic alterations during colorectal tumorigenesis. Recent studies found that fusion of normal chromosome 18 in colorectal carcinoma cell lines inhibits their tumorigenicity, providing additional evidence that chromosome 18 may harbor a tumor-suppressor gene that is inactivated in colorectal tumorigenesis.[31] We identified a candidate tumor-suppressor gene on chromosome 18q that appears to be altered in colorectal cancer.[32] Using a panel of several polymorphic DNA probes to study tumors which have lost some, but not all of 18q, we identified a common region of deletion centered at 18q21.3. One anonymous probe in this region detected homozygous loss of sequences in a primary colorectal carcinoma. Homozygous losses are rare and are thought to be a hallmark of tumor-suppressor genes. Using this probe as a starting point, we used a bidirectional chromosome walking strategy to clone 370 kilobases of contiguous genomic DNA in the deleted region thought to harbor the tumor-suppressor gene target. Potential exons in the cloned region were identified by cross-species hybridization at reduced stringency, followed by a comparison of human–rodent sequence identities in which candidate exons contained long open reading frames flanked by appropriate splice donor and acceptor sites and lariat sequences. The expression of these potential exons was identified using an "exon-connection" strategy based on the polymerase chain reaction. Using standard cloning techniques, we eventually obtained a complementary DNA from the gene (called *dcc*, for deleted in colorectal carcinomas). The *dcc* gene encodes a putative translation start site, signal peptide, and hydrophobic transmembrane region dividing the predicted protein into extracellular (1100 amino acid) and intracellular (324 amino acid) domains. Preliminary data suggest that tissue-specific *dcc* transcripts may be generated through alternative splicing of the primary transcript. The coding region is composed of at least 28 exons and appears to be flanked by lengthy 5′ and/or 3′ untranslated regions, accounting for a significant portion of the 10–12-kilobase *dcc*-encoded message. The predicted amino acid sequence of the extracytoplasmic portion of the protein has significant sequence similarity to neural cell adhesion molecules and other related cell-surface glycoproteins. By mediating cell–cell and cell–substrate interactions, this class of molecules is thought to be important in mediating cell growth and differentiation. Alterations of the *dcc* gene may interfere with maintenance of these critical controls and play a role in the pathogenesis of human colorectal neoplasia.

Several alterations of the *dcc* gene were identified in colorectal tumors, suggesting that this gene is an excellent tumor-suppressor gene candidate. Using polymerase chain reaction techniques, *dcc* gene expression was identified in most normal tissues studied, including normal colonic mucosa. By contrast, expression of the same portion of the *dcc*-encoded message was absent or reduced in 15 of 17 colorectal carcinoma cell lines. Somatic alterations of *dcc* also have been identified in several primary tumors, colorectal carcinoma xenografts, and colorectal carcinoma cell lines. Demonstration of a functional tumor-suppressor effect should provide additional support for *dcc* as a tumor-suppressor gene. Recently, transfection studies have shown that the wild-type *p53* gene specifically can suppress the growth of human colorectal carcinoma cells in vitro and that an in vivo derived mutation in the *p53* gene abrogates this suppressing ability.[33] We recently completed construction of full-length *dcc* expression vectors to use in similar ongoing transfection studies.

Allelic deletions on chromosome 5q have been found in almost 40% of carcinomas and sporadic adenomas studied.[7,34] The gene responsible for familial adenomatous polyposis was mapped to the deleted region.[35,36] A candidate tumor-suppressor gene[37] located at chromosome 5q21 recently was identified (the *mcc* gene, for mutated in colorectal cancers). Alterations in *mcc* include one tumor with somatic rearrangement of one *mcc* allele and several tumors with somatically acquired point mutations in the *mcc* coding region. A partial *mcc* complementary DNA has been cloned. This DNA encodes an 829 amino acid protein with a short region of sequence similarity to the G-protein-coupled m3 muscarinic acetylcholine receptor. The region of sequence similarity coincides with that region of the receptor that recently was shown to be critical for G-protein activation.[38] Members of the G-protein family are believed to be important in transducing signals in the cell. The connection between *mcc* and the G-protein-activating region of the receptor is intriguing in light of previous studies showing the importance of G-proteins

in neoplasia; however, it remains to be shown whether *mcc* actually interacts with G-proteins in vivo. Additional studies are needed to elucidate the relationship between *mcc* gene mutations and sporadic colorectal cancers and to determine whether *mcc* is mutated in the germline of patients with familial adenomatous polyposis.

In studying the genetic alterations occurring in colorectal tumorigenesis, several pertinent points currently are evident. First, it appears that the accumulation rather than the order of genetic changes is the most important. Although DNA hypomethylation, *ras* gene mutation, and 5q allelic loss tend to occur relatively early during tumor progression with 18q/17p losses relatively late, the sequence of events is not absolute. Second, genetic alterations probably continue to accumulate after carcinomas have been produced, and this accumulation may parallel clinical progression. This multiplicity of genetic changes must be considered in any model of the genetic origin of human neoplasia and should prompt caution in overinterpreting the significance of any one alteration. Finally, colorectal carcinogenesis is a complex process in molecular terms. Although deletions of 17p, 18q, and 5q occur frequently, deletion of other chromosomes often are found even in tumors with chromosomal 5, 17, and 18 loss. The other chromosomes probably contain tumor-suppressor genes (or oncogenes) in addition to the ones we discussed. The complexity of genetic changes in human neoplasia may be reflected in the biologic heterogeneity of these tumors.

In summary, current data suggest a model of colorectal tumorigenesis in which mutational activation of a dominant-acting oncogene occurs in concert with inactivation of several tumor-suppressor genes to produce progressive tumorigenesis. Studies of these genes should provide additional understanding of the molecular pathogenesis of a common human neoplasm. We hope the lessons learned will be useful for other tumors of epithelial origin.

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