Refining Molecular Analysis in the Pathways of Colorectal Carcinogenesis

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Background & Aims: **In the stepwise model, specific genetic and epigenetic changes accumulate as colorectal adenomas progress to carcinomas (CRCs). CRCs also acquire global phenotypes, particularly microsatellite instability (MSI) and aneuploidy/polyploidy (chromosomal instability, CIN). Few changes specific to MSI-low or CIN+ cancers have been established.** ***Methods:*** **We investigated 100 CRCs for: mutations and loss of heterozygosity (LOH) where appropriate, of *APC*, K-ras, *BRAF, SMAD4*, and p53; deletion on 5q around *APC* and 18q around *SMAD4*; total chromosomal-scale losses and gains; MSI; and CIN.** ***Results:*** **As expected, CIN− cancers had fewer chromosomal changes overall than CIN+ lesions, but after correcting for this, 5q deletions alone predicted CIN+ status. 5q deletions were not, however, significantly associated with *APC* mutations, which were equally frequent in CIN+ and CIN− tumors. We therefore found no evidence to show that mutant *APC* promotes CIN. p53 mutations/LOH were more common in CIN+ than CIN− lesions, and all chromosomal amplifications were in CIN+ tumors. CIN− cancers could be subdivided according to the total number of chromosomal-scale changes into CIN-low and CIN-stable groups; 18q deletion was the best predictor, being present in nearly all CIN-low lesions and almost no CIN-stable tumors. MSI-low was not associated with CIN, any specific mutation, a mutational signature, or clinicopathologic characteristic.** ***Conclusions:*** **Overall, the components of the stepwise model (*APC*, K-ras, and p53 mutations, plus 18q LOH) tended to co-occur randomly. We propose an updated version of this model comprising 4 pathways of CRC pathogenesis, on the basis of 5q/18q deletions, MSI (high/low), and CIN (high/low/stable).**

The adenoma-carcinoma sequence for the histologic development and progression of colorectal cancer (CRC) is well established,[1] as is the superposition of a series of acquired genetic changes onto this sequence.[2] It is generally held that early colorectal adenomas initially acquire 2 *APC* mutations, often followed by mutation of the oncogene K-ras, loss of chromosome 18q, and mutation/loss of the p53 tumor suppressor.[3] This stepwise model of tumorigenesis is primarily based on observations of mutation frequencies in unselected bowel tumors of various stages, grades, and sizes.

The finding that many CRCs have acquired some form of genomic instability has added complexity to the stepwise model. In most cases, instability is essentially a global (or phenotypic) trait with no known underlying genetic defect. The only exception is defective mismatch repair owing to *MLH1* promoter hypermethylation.[4] This results in an increased tendency to slippage in short repeat sequences, manifest as microsatellite instability (MSI-high phenotype) and frameshift mutations in genes such as *BAX* and *TGFBR2*.[5,6] Other CRCs reportedly have low-level MSI, although its origin and importance are unclear.[7,8] It is not known, for example, whether frameshift mutations occur more frequently in MSI-low cancers.[9]

Another form of genomic instability occurs at the chromosomal level and is termed chromosomal instability (CIN), a poorly defined phenomenon, the existence of which is often inferred from the finding of aneuploidy and/or polyploidy in cancers.[10] It has been proposed that p53 mutations cause CIN,[11,12] although others have disputed this. More recent data from mouse embryonic stem cells have suggested that inactivation of *APC* itself might lead to CIN, although gross instability was only detected when anti-apoptotic mechanisms were inactivated.[13] We have shown that gross CIN is not present in benign colorectal tumors with *APC* mutations,[14] but it has remained possible that the phenomenon only be-

Abbreviations used in this paper: aCGH, array comparative genome hybridization; CIMP, CpG island methylator phenotype; CIN, chromosomal instability; CRC, colorectal cancer; LOH, loss of heterozygosity; MSI, microsatellite instability.

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comes apparent in malignant lesions that have deficient mitotic checkpoints.

The stage of tumorigenesis at which the MSI and CIN phenotypes arise and can be distinguished is controversial, with some stating that they initiate and are essential for tumorigenesis[15] and others arguing that genetic instability is usually acquired at or after progression from adenoma to carcinoma.[16] The finding that some CRCs have neither MSI-high nor CIN[17] suggests either that genomic instability is nonessential for growth of this malignancy, or that a third type of instability exists.

Superimposed on the genetic pathways of colorectal tumorigenesis is an epigenetic pathway, the CpG island methylator phenotype (CIMP),[18] in which some CRCs tend to silence gene expression by promoter methylation. CIMP+ cancers have been reported to be associated with the MSI-high phenotype, but non−MSI-high CIMP+ cancers are not uncommon.[19] It has been suggested that, rather like MSI-low, CIMP+ cancers do not form a discrete group, but rather that CIMP is a continuous trait,[20] although this conclusion is strongly disputed.[21]

We wished to obtain a comprehensive profile of a set of CRCs as regards basic clinicopathologic data, genotype (the molecular components of the stepwise molecular model), and phenotype (MSI and CIN). Our aims were to test the stepwise model and, if appropriate, to set up our own model of the pathways of colorectal carcinogenesis. We therefore screened a set of 100 CRCs for mutations of *APC*, p53, *SMAD4*, K-ras, and *BRAF* and for allelic loss (loss of heterozygosity [LOH]) at the first 3 of these loci. We added data on MSI-high and MSI-low status and CIN, and assessed deletions on 5q and 18q, involving *APC* and *SMAD4*, respectively.

Methods

Samples and Baseline Molecular Data

An unselected series of 100 fresh-frozen CRCs and paired normal bowel was obtained from St Mark's Hospital, London; fixed tissue was obtained from the same tumors. By routine histology, all cancers contained more than 60% neoplastic cells. Clinicopathologic data were obtained from hospital records: age; sex; location (proximal or distal); Dukes stage (A, B, C); Jass score[22] (1, 2, 3, 4); and grade (well-, moderately, or poorly differentiated). DNA was extracted from each sample by using standard methods. Samples were studied on an anonymous basis as approved by Harrow Local Research Ethics Committee.

Mutation Screening

Cancers were screened for *APC* mutations using fluorescent−single-strand conformation polymorphism and denaturing high-performance liquid chromatography analyses so as to cover the coding sequence and intron-exon boundaries before codon 1600. p53 (exons 5−8) was screened using single-strand conformation polymorphism analysis based on silver-stained midi-gels. The coding region of *SMAD4* was screened by denaturing high-performance liquid chromatography analysis. In all cases, details are available from the authors. For each of these 3 genes, any sample with a bandshift was sequenced for the appropriate fragment in forward and reverse orientations from a new polymerase chain reaction product. Mutations at K-ras (codons 12, 13, and 61) and the *BRAF* mutation hotspot at codon 600 were detected using direct sequencing in forward and reverse orientations (details available from authors).

Loss of Heterozygosity Analysis

LOH was assessed at the *APC*, p53, and *SMAD4* loci (the last of these also referred to as 18q LOH for consistency with the stepwise model). Microsatellites close to each locus were typed in each cancer and constitutional DNA (D5S346, D5S656, D5S2001, and D5S489 for *APC*; D17S796, TP53.CA, and D17S786 for p53; D18S46, D18S474, D18S484, and DCC for *SMAD4*). Constitutionally homozygous markers or markers showing MSI were scored as noninformative. Otherwise, at each marker, LOH was considered to be present if the area under one allelic peak in the tumor was less than 0.5× or greater than 2× that of the other allele, after correcting for the relative allelic areas using the constitutional DNA. If there was any discordance among markers, the marker(s) closest to the gene of interest were given precedence in classifying the cancer.

Array Comparative Genomic Hybridization

Because LOH can occur by mechanisms that do not alter gene dosage (Figure 1), deletions of chromosomes 5q (around *APC*) and 18q (around *SMAD4*) were also determined from array comparative genomic hybridization (aCGH) data,[23] which were already available from 54 cancers and which were obtained on 10 additional cancers using reported methods.[24] We formally required loss of at least 2 clones flanking the *APC* and *SMAD4* genes to score deletion at these sites ($\log_2$ tumor: normal ratio <-0.2) by aCGH, although, in practice, a minimum of 5 adjacent clones, and usually a much larger region, was deleted. The total number of chromosomal-scale changes (gains and deletions) in each cancer was also counted, according to criteria described.[23]

Chromosomal Instability Status

CIN status was determined by flow cytometry as described.[14] In short, cancers with an aneuploid and/or polyploid flow cytometry peak distinct from the diploid peak and corresponding to a DNA index of >1.2 were classed as CIN+.

Microsatellite Instability Analysis

The MSI status (high/low/stable) of each CRC had been reported[25] for all but 6 cancers. We had shown MSI-low to be a quantitative trait, with evidence for an extra, super-