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Potential Roles of Genetic Biomarkers in Colorectal Cancer Chemoprevention

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Abstract Colorectal cancer is a significant cause of morbidity and mortality in industrialized societies and the second most frequent cause of cancer death in the United States. Surrogate endpoint biomarkers are gaining wide acceptance in early diagnosis and short-term cancer chemoprevention trials in place of cancer endpoints. Molecular genetic biomarkers can be useful tools in identifying subjects at risk of developing cancer and screening for early cancers amenable to complete cure. They may be useful both in predicting and assessing response to a given therapy and in determining prognosis after an initial diagnosis has been made. Ideally, biomarkers should fulfill some, if not all, of the following criteria: variability of expression between phases of carcinogenesis, association with cancer risk, ability to undergo modification in response to a chemopreventive agent, and finally, permit ease of measurement. In consideration of colorectal cancer chemoprevention, several genetic biomarkers seem to meet many of these criteria, as they do exhibit distinct variability of expression at different phases of carcinogenesis, are often strongly associated with increased cancer risk (especially the hereditary/familial syndromes), are generally able to be measured relatively easily through peripheral blood sampling (germline mutations) or by colonic mucosal sampling by endoscopic techniques (somatic mutations). In some cases, genetic biomarkers have also been demonstrated to undergo modification in response to a chemopreventive agent. With further understanding of the genetic and molecular changes involved in sporadic and familial colorectal carcinogenesis, genetic biomarkers appear to hold great potential for the identification of subjects at high risk of developing colorectal cancer, as well as the development of novel chemopreventive approaches and form a promising area for further research. J. Cell. Biochem. Suppl. 34:28–34, 2000.
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Colorectal cancer (CRC) is an ideal cancer on which to target prevention efforts. An estimated 130,000 new cases arise every year and, despite advances in detection and treatment, approximately 55,000 people die of CRC annually, making it the second most frequent cause of cancer death in the United States [Landis et al., 1999; Rustgi, 1994]. Prognosis largely depends on the pathological stage at diagnosis, with 5-year survival rates of greater than 90% when CRC is limited to the mucosa, compared with less than 10% for metastatic disease [Crucitti et al., 1991]. The progression from normal colonic mucosa to invasive cancer is a process that takes many years; it can be interrupted by the removal of neoplastic adenomatous polyps. Therefore, any intervention that can prevent the development of CRC, delay its progression, or lead to cancers detected at an earlier stage can have a marked impact on the mortality associated with CRC.

During the past decade, there have been major advances in our understanding of CRC genetics. In addition, several novel potential chemoprevention agents in various stages of development may be beneficial in the prevention of CRC. This review focuses on potential methods of taking advantage of our increasing knowledge of CRC genetics to optimize chemoprevention studies, from several different per-

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spectives: by consideration of hereditary colon cancer syndromes associated with extremely high risk of cancer, cohorts at moderately increased risk of cancer, and finally, somatic genetic events associated with the development of CRC. Thus, some of the advantages and disadvantages of a genetic approach to chemoprevention of colorectal cancer are highlighted.

COLORECTAL CANCER GENETICS

The majority (80%) of CRC cases are sporadic; the remainder have a familial component [Marra and Boland, 1995]. Invasive CRC is preceded by several transformations, from normal to adenomatous mucosa to invasive carcinoma, a process that evolves over several years. Multistep models of CRC carcinogenesis have identified genetic mutations that parallel morphologic changes from normal epithelium, to small polyp, to large polyp and carcinoma [Gryfe et al., 1997; Toribara et al., 1995]. Genetic changes important in colorectal carcinogenesis include general alterations in cellular DNA content (aneuploidy or DNA index), nuclear aberrations, and altered patterns of gene expression. The more specific genetic alterations are best illustrated by Vogelstein's model of colorectal carcinogenesis [Fearon and Vogelstein, 1990], which indicates that development of cancer in the gut epithelium is likely the result of the successive accumulation of multiple genetic mutations. The series of genetic alterations (Table I) involved in CRC carcinogenesis [Greenwald et al., 1995] identified thus far include the K-*ras* oncogene on the short arm of chromosome 12; several tumor-suppressor genes, including the p53 gene at chromosome 17q [Fearon and Vogelstein, 1990] the *APC* gene on chromosome 5q, possibly the *DCC/DPC4* [Walsh, 1997; Thiagalingam et al., 1996] gene on chromosome 18q; and the mismatch repair (MMR) genes *hMSH2* (2p21), *hMLH1* (3p21), *hPMS1* (2q31–33), *hPMS2* (7p22) and *hMSH6* (2p21). Mutations in the *APC* gene and MMR genes occur relatively early in the development of colorectal tumors, whereas mutations of p53 are relatively late events in the pathway. The accumulation of genetic lesions is more important than the presence of any single alteration [Fearon and Vogelstein, 1990]. For most of those individuals with a family history, CRC risk is probably a complex interaction of genetic and environmental factors. However, in a subset of individuals, increased susceptibility to CRC is inherited through a single gene mutation. Therefore, in hereditary colorectal cancer syndromes, an inherited germline mutation leads to increased susceptibility for subsequent alterations, whereas in sporadic forms of cancer, the alterations are acquired somatically. Hereditary colorectal cancer can be divided into two major categories: the familial adenomatous polyposis (FAP) syndromes and hereditary nonpolyposis colorectal cancer (HNPCC). The experience derived from these entities has been instrumental in advancing our understanding of the molecular basis of CRC carcinogenesis.

TABLE I. Potential Genetic Biomarkers in Colorectal Cancer and Their Genetic Loci

Gene	Locus
K-*ras*	12p12
DCC	18q21
APC	5q21
hMSH2	2p21
hMLH1	3p21
hMSH6	2p21
hPMS1	2q31–33
hPMS2	7p22
p53	17p53

FAP is an autosomal dominant syndrome associated with mutations of the adenomatous polyposis coli gene (located on chromosome 5q21) [Bodmer et al., 1987], which result in the development of hundreds of polyps by the third decade of life. CRC inevitably occurs unless the colon is prophylactically removed. Surgical options for patients affected with FAP include total proctocolectomy with ileoanal anastomosis or subtotal colectomy with ileorectal anastomosis. When the latter operation is performed, patients must continue to undergo surveillance sigmoidoscopy of the remaining rectal segment every 6 months, with removal of any new polyps that have formed.

HNPCC is more common than FAP (accounting for 4–6% of all CRCs) and is a much more heterogeneous syndrome. Affected individuals are at markedly increased risk of developing CRC (with lifetime risks of 70–90% [Vasen et al., 1996; Offit, 1998], and of developing multiple other tumors such as endometrial, extracolonic gastrointestinal, and genitourinary tumors. The family history is the key to diagnosing HNPCC because affected individuals have similar numbers of polyps as the general population. Because of the heterogeneity of the syn-

drome, an international collaborative group devised the Amsterdam criteria [Vasen et al., 1991] for the diagnosis of HNPCC, which require three or more relatives with verified CRC, one of whom must be a first-degree relative of the other two; CRC spanning two or more generations; and at least one CRC diagnosed before 50 years of age. In addition, several other clinical criteria, such as the Modified Amsterdam criteria or the Bethesda criteria [Rodiguez-Bigas et al., 1997] have been developed because of concerns that the Amsterdam criteria were too restrictive, especially for small families and where extracolonic HNPCC tumors exist. The recent isolation of several DNA mismatch repair genes (*hMSH2, hMLH1, hPMS2,* and *hMSH6*) associated with the HNPCC syndrome has made it possible to identify carriers of a mutated gene within a family [Leach et al., 1993; Papadopoulas et al., 1994; Nicolaides et al., 1994].

A specific mutation in the *APC* gene, termed I1307K, has recently been associated with an increased risk of developing colorectal cancer in the Ashkenazi Jewish population. Individuals who inherit this particular mutation do not have the polyposis phenotype but are at increased risk of sporadic CRC. The precise CRC risk with the mutation remains uncertain; in the initial report, the relative risk varied from 1.5 to 6.5, depending on other factors in the patient's personal and family history [Laken et al., 1997]. A recent study confirmed the association, but estimated the relative risk for CRC to be 1.5–1.7 [Thiagalingam et al., 1996]. This mutation represents a novel paradigm for inherited cancer predisposing genetic abnormalities, as individuals who inherit this particular mutation appear to be at moderately, rather than markedly, increased risk of the development of cancer.

USE OF GENETIC BIOMARKERS IN CRC CHEMOPREVENTION TRIALS

The use of genetic biomarkers in colorectal cancer chemoprevention studies must be governed by the same principles that apply to the use of other biomarkers in clinical trials. Several criteria have been proposed (Table II) to identify the ideal biomarker [Einspahr et al., 1997]. The utility of biomarkers in chemoprevention trials can be broadly grouped in two main categories: (1) to identify subjects at high risk of developing cancer, as part of studies in which the primary endpoint is cancer incidence; and (2) to serve as a substitute or surrogate endpoint, with trials studying the effect of the preventive strategy on the marker, rather than the cancer [Mark et al., 1996]. The following discussion reviews how genetic biomarkers fulfill the proposed criteria for ideal biomarkers.

TABLE II. Criteria for an Ideal Biomarker and Genetic Biomarkers in CRC

Criterion	Example	References
Variability of expression between phases of carcinogenesis	Mutations in the *APC* and MMR genes occur early in the pathway	[Thiagalingam et al., 1996; Gryfe et al., 1997]
	p53 and *DCC* mutations occur relatively later	
Associated with risk of developing cancer	Lifetime CRC risks of ~70–100% with MMR and *APC* gene mutations	[Offit, 1998; Aarnio et al., 1999]
Ability to undergo modification in response to a chemopreventive agent	↓ Mutagenesis—Oltipraz	[Kelloff et al., 1997]
	↑ Apoptosis—NSAIDS	
	↑ Differentiation—4HPR	
	↓ Proliferation—DHEA	
	↓ p21 *ras* protein—piroxicam	[Singh et al., 1993]
	↓ p53 protein—ibuprofen	[Crist et al., 1995]
	↓ aberrant crypt foci—sulindac	[Takayama et al., 1998]
Easily assayed	Germline mutations can be determined by peripheral blood analysis	[Luce et al., 1995; Kopreski et al., 1997]
	Colonic tissue (sporadic CRC) can be relatively easily obtained by endoscopic techniques	[Toribara and Sleisenger, 1995]

APC, adenomatous polyposis coli; MMR, mismatch repair; *DCC,* deleted in colon cancer gene; CRC, colorectal cancer; NSAIDS, nonsteroidal anti-inflammatory drugs; 4HPR, all-*trans*-N-(4-hydroxy)retinamide; DHEA, Dehydro epiandrosterone.

Variability of Expression Between Phases of Carcinogenesis

The first requirement of the ideal biomarker is that there must be variability of expression of the biomarker between phases of carcinogenesis. We are fortunate that this variability has been extremely well characterized for colorectal cancer. In general, the progression of genetic events may be used to monitor the stages of this process, which can subsequently be exploited both for the identification of high-risk groups or as targets for chemoprevention agents. It is likely that the use of multiple rather than single molecular markers (i.e., *APC*, MMR, and p53) will prove most useful to assess the temporal phases of carcinogenesis.

Associated Risk of Developing Cancer

The second requirement for an ideal biomarker is that it must be associated with risk of developing cancer. Individuals with *APC* or mismatch repair gene mutations, for example, fulfill this criterion, which permits the identification of high-risk cohorts for chemoprevention trials. The greatest advantages of using high-risk cohorts such as those individuals with hereditary colorectal cancer include estimates of cancer penetrance as high as 70–90% [Offit, 1998; Thiagalingam et al., 1996] in affected individuals, which can significantly decrease sample sizes necessary for clinical studies [Burt, 1996]. Prevention trials generally require extremely large patient numbers to have sufficient power to test the efficacy of an intervention. The standard phase III trial studying cancer incidence as an endpoint requires sample sizes in the thousands and follow-up duration in excess of 5 years; incurring extremely high costs. The number of subjects required for a study and/or the duration of the study decreases as the frequency of the outcome under study increases. The study of individuals affected with familial polyposis have been instrumental in advancement of our knowledge of the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) as chemopreventive agents and FAP patients will surely will be key subjects in trials of future novel agents. FAP has provided a useful model in that the formation, recurrence, or regression in number and size of adenomas represents an endpoint that can be studied with great facility primarily because of the multiplicity of polyps and their relatively rapid formation. The fact that FAP patients who have undergone subtotal colectomy require biannual sigmoidoscopy of the rectal remnant for removal of new polyps permits a relatively rapid assessment of the efficaciousness of chemopreventive agents. Also, recent studies by Bertagnolli and colleagues [Mahmoud et al., 1999]. suggest that the effectiveness of chemopreventive agents in preventing *Apc*-related (murine) tumor formation may depend on the type of mutation in the *APC* gene.

The genetic characterization of HNPCC offers the potential of another high-risk target group. Although HNPCC is an attractive cohort to target for chemoprevention, controversies in eligibility criteria constitute issues of substantial difficulty in study design, which may limit recruitment of adequate numbers [Kelloff et al., 1996; Dhingra et al., 1993]. The precise definition of HNPCC remains controversial. Consequently there is a paucity of data relating to chemoprevention trials. However, a recent in vitro study by Ruschoff et al. [1998] showed that microsatellite instability in colorectal cancer cells associated with mutations of the mismatch repair (MMR) genes *hMLH1, hMSH2,* and *hMSH6* is markedly reduced during exposure to aspirin or sulindac. These findings suggest that aspirin/sulindac may provide an effective prophylactic therapy for individuals with the HNPCC syndrome associated with these MMR genes.

Individuals at high risk of cancer are likely to be highly motivated to decrease cancer risk. This group may be particularly open to study participation perhaps because of patient awareness of the extremely high risk of cancer and the fact that the only alternative to a potentially effective study drug could be major surgery (i.e., conversion of a subtotal colectomy to a total colectomy with ileoanal anastomosis for an FAP patient.) One of the disadvantages of including patients with hereditary colorectal cancer as subjects in chemoprevention trials is the limitations on generalizing the results to the far more common forms of sporadic colorectal cancer [Burt, 1996; Hamilton, 1992; Friend, 1990]. A means of circumventing the problem of limitations in generalizability may be to target larger populations found to be at moderately elevated risk. Examples of this approach can be found in several ongoing trials studying the effect of chemoprevention agents in individuals with a history of CRC or adenomatous polyps. The I1307K mutation, the finding having been substantiated by other investigators as well

[Gryfe et al., 1999], may serve as an ideal genetic biomarker to identify a cohort at moderately elevated risk of the development of sporadic CRC for chemoprevention trials. One of the main outstanding issues is the relative risk associated with inheritance of the I1307K mutation; if the relative risk is 1.5–1.7 as estimated by recent reports, there may not be sufficiently increased risk to have a significant impact on the decreasing sample sizes necessary for clinical trials.

The use of somatic alterations is another potential method for targeting high-risk groups, with the caveat that one should be careful to choose alterations sufficiently early in the process to allow chemopreventive agents to have effect. The earlier a genetic marker appears in the carcinogenic process, the greater the chance that a successful intervention will result in decreased cancer risk. Using this principle implies that alterations in APC, for example, which occur early in the CRC carcinogenic pathway, may be useful to identify cohorts for study participation, whereas p53, which is a late-stage alteration, may be a more useful endpoint biomarker.

Modifiable With a Chemopreventive Agent

The advantage of using surrogate biomarkers as study endpoints is that they reduce the time necessary to conduct the trial. To fulfill this role, the biomarker must be modifiable with the chemopreventive agent being studied. Only then can the markers be used as surrogate endpoints in chemopreventive drug development. All carcinogens that could cause mutations in the *APC*, p53, *hMLH1*, and *hMSH2* and K-*ras* genes are potential targets for chemopreventive agents [Mahmoud et al., 1999]. Specific agents such as oltipraz prevent carcinogenic compounds from reaching or reacting with critical target sites by inhibiting the metabolic activation of carcinogens catalyzed by P-450, amplifying detoxification systems, and trapping carcinogens before they reach critical target sites. Agents that suppress promotion prevent evolution of the carcinogenic process in cells that would otherwise become malignant include differentiating agents (i.e., retinoids), inhibitors of oncogene action (i.e., terpenes), selective inhibitors of cell proliferation (i.e., DFMO, calcium), and NSAIDs. Antioxidants such as β-carotene, vitamin E, and curcumin have the potential to inhibit mutations in the genes known to be associated with colorectal tumorigenesis by their ability to scavenge free radicals and terminate lipid peroxidation. Finally, agents such as D-limolene, which is a blocking agent, an antiproliferative, and an antioxidant, has the potential to target multiple genetic or cellular events [Greenwald et al., 1995].

The ability of chemopreventive agents to modulate surrogate endpoint biomarkers has been demonstrated by several reports. For example, Singh et al. [1993] have shown that dietary piroxicam/DFMO in rats can significantly suppress expression of the azoxymethane (AOM)-induced p21 *ras* protein (the protein product of cellular *ras* proto-oncogenes) during the development of AOM-induced colon carcinogenesis in male F344 rats. Another study, conducted by Crist et al. [1995], found a decrease in accumulation of the p53 protein, as well as a reduction of K-*ras* mutations at codon 12, in the AOM rat model treated with piroxicam and ibuprofen. Similarly, it has been shown that aberrant crypt foci of the colon seen on endoscopy may be precursors of adenoma and carcinoma [Takayama et al., 1998]. Furthermore, it has been shown that, after NSAID (sulindac) therapy, the number of these foci decreases, suggesting their potential role as biomarkers for the diagnosis and chemoprevention of CRC.

Easily Assayed

Biomarkers must be easily assayed—ideally using noninvasive methods—and be highly sensitive and specific. The presence or absence of germline mutations can be relatively easily determined by analysis of peripheral blood, a major advantage to their use. However, genetic analysis can be both time-consuming and expensive, especially in syndromes such as HNPCC, characterized by multiple associated genes with mutations scattered throughout the genes. When considering the use of genetic biomarkers in sporadic CRC, colonic tissue samples must be obtained for analysis, since sporadic mutations occur in premalignant or malignant tissue only, and not in the germline. Compared with other organs, endoscopic techniques facilitate the acquisition of colonic tissue for histologic and molecular examination during flexible sigmoidoscopy or colonoscopy. Because procedures are part of standard care for many patients (sigmoidoscopy as screening for the general population, and colonoscopy for surveil-

lance of the population at moderately increased risk of CRC), the use of genetic biomarkers is feasible for CRC prevention trials.

Reproducible and Reliable

Finally, like other biomarkers, measurement of genetic biomarkers should be easily reproducible, with minimal interexperimental variation to permit longitudinal follow-up evaluation. In the first phase of considering a possible biomarker, one attempts to compare the prevalence of the putative marker in malignant and nonmalignant tissue within the same patient. It is extremely important to establish a baseline for the expression of any given marker in normal-risk epithelium. Many studies rely on microdissection to improve the likelihood of determining genetic alterations associated with the formation and progression of adenomas. Reproducibility of replicate measurements, even from the same adenoma samples, may be problematic because of heterogeneity within these premalignant lesions. Some genetic alterations, such as mutations of the p53 gene, are measured by immunohistochemical detection of protein in tissue sections. One report showed a loss of immunostaining intensity over a matter of weeks, with some cases becoming p53 negative [Jacobs et al., 1996]. Interpretation of existing studies using genetic biomarkers is complicated by variability of the reported studies, which are affected by differences in degree of dysplasia, small sample size, and differences in study populations. These types of studies stress the importance of knowing how sample handling and storage affect each genetic biomarker.

CONCLUSIONS AND FUTURE PROSPECTS

Genetic biomarkers appear to be valuable tools as surrogate endpoint biomarkers in the early detection and assessment of both progression and regression of colorectal cancer. They satisfy many of the criteria for the ideal biomarker: (1) they exhibit variability of expression along the different phases of carcinogenesis; (2) they are associated with increased cancer risk; (3) they are relatively easily measured by peripheral blood sampling or by endoscopic techniques; and (4) they have been demonstrated by some studies to undergo modification in response to treatment with a chemopreventive agent. The identification of the various mismatch repair genes and our understanding of microsatellite instability in HNPCC have contributed to our understanding of the genetics of the syndrome and have great potential as genetic biomarkers in the CRC chemoprevention studies. The best understood of the familial syndromes unfortunately also happens to be the least common, namely FAP. However, although remarkable progress has been made in identifying the genetic events important in CRC, further work is necessary to determine the reproducibility of biomarker measurements, their precise associations with cancer risk, and the generalizability of biomarker studies to conclusions about cancer incidence. With further elucidation of the various genetic changes involved in CRC carcinogenesis, possibly involving a series or panel of mutations, genetic biomarkers appear to hold promise in identifying subjects at high risk of developing CRC, as well as in developing newer chemopreventive strategies. Genetic biomarkers warrant intense research in this relatively new and exciting approach in the prevention of cancer.

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