Journal of Cellular Biochemistry, Supplement 16G:151-157 (1992)

Modulation of Gene Expression as a Biomarker in Colon

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Abstract Computer-driven scanning and image processing methodology has demonstrated that genetic inheritance of risk for colorectal cancer in familial polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) families is associated with highly pleiotropic effects on patterns of gene expression in the flat colonic mucosa. The mitochondrial (mt) gene encoding subunit 3 of cytochrome oxidase (COXIII) is one of a panel of cloned sequences which characterize genetic risk. Expression of COXIII decreased in progression of, and risk for, colonic tumors *in vivo*. Further, metabolizable, unbranched, short-chain fatty acids (SCFAs) elevated expression of mtCOXIII, as well as mtCOXI, in HT29 cells and also elevated mtCOX enzymatic activity. However, expression of nuclear encoded COX subunits were unaffected. These changes may be related to documented alterations in mitochondria structure and function in transformed colonic epithelial cells.

SCFAs produced when colonic microflora causes fermentation of fiber are the principle energy source for normal colonic epithelial cells; SCFAs also induce a more differentiated phenotype both *in vitro* and *in vivo*. Therefore, a mechanistic link may exist between molecular events in inherited risk and a dietary factor (fiber) which may modulate such risk.

In a preliminary intervention trial in collaboration with M. Lipkin, high risk HNPCC patients received daily supplements of 1500 mg $CaCO_3$ per day, which may be protective for development of colorectal tumors. Elevations in COXIII expression were seen in 7 of 12 patients within the first 7 months, followed by complex changes in expression of this sequence.

Key words: calcium, chemoprevention, colorectal cancer, fiber, gene expression, genetic risk, intermediate biomarker, mitochondria, short-chain fatty acids

The development of colorectal cancer is characterized by the accumulation of multiple genetic abnormalities. These include high frequency of mutations in Ki-*ras*2, deletions and mutations in the p53 gene, deletions of the DCC gene [1], and mutations at the APC and MCC loci [2-5]. Low to modest amplifications of the c-*myc* gene also are frequent in colonic tumors [6, and unpublished]. The term "tumor allelotype" has been coined to describe not only the common alterations which may be the sine qua non of colorectal cancer, but also the extensive number of loci (perhaps hundreds to thousands) which are deleted or altered throughout the genome in a heterogeneous pattern in most colonic cancers [7], and which may interact with other complex changes in gene regulation to produce the overall tumor phenotype [8-10].

The strides in detecting and dissecting these genomic alterations are important because they offer insight into both the mechanisms responsible for tumor initiation and progression and also because they provide understanding and reagents which may be the key to new methods of prevention, detection, prognosis and treatment. These clinical applications are being pursued and practical approaches will probably be clarified in the near future. The mechanistic implications of the genetic alterations are clear: most carcinogens are mutagens, and the transformed state is certainly transmissible from cell generation to generation. Thus, the initiating events and other alterations which impart the fundamental properties of the tumor cell probably reside among the physically altered genetic material [1,3].

Besides the numerous structural genomic alterations which characterize tumors, alterations in the level of gene expression are also very common in transformed cells and neoplastic growth [9]. These changes can involve up to 10% of the expressed genes, which is equivalent to 1000 sequences, even in simple model systems of

retroviral transformation in culture [11]. The importance of these changes, though complicated by the contribution of changes in growth rate and cellular heterogeneity within tumors in vivo, should not be underestimated. First, the level of expression may in part determine the penetrance of a structurally altered locus. Second, the effects of structurally altered loci may be imparted by pleiotropic effects on the expression of many sequences. For example, the product of the p53 gene may function as a transcription factor [12-14], and mutations in p53 may initiate a cascade of effects on gene expression. Third, normal development proceeds through the stable differentiation of a multitude of cell types which, as far as is presently understood, depend upon differential gene regulation rather than structural alterations. This, along with the clear demonstration that tumor cells can be induced to differentiate under certain circumstances, demonstrates that many stably inherited and transmissible aspects of cell phenotype must be dependent on reversible alterations in gene function.

EXPERIMENTAL APPROACH

While many changes in expression of individual genes can be readily documented in colonic cancer, we wished to understand the extent of, and interaction among, these changes. In order to do this, we developed a scanning and image processing system with which we could quantitate the level of expression of each of thousands of cloned sequences in a reference cDNA library [8-10]. This methodology was used to develop a large data base on the levels of expression of each sequence in biopsies of normal mucosa, adenoma, and carcinoma. As expected, there was a progressive increase in the number of alterations in gene expression in progression of the disease, amounting to about 7% of the total sequences assayed in carcinoma compared to normal flat mucosa [8].

GENETIC RISK GROUPS

The normal mucosa used in the above comparisons was from individuals with no colonic or other cancer for several generations [8]. This tissue was also compared to the normal appearing flat mucosa from individuals with familial adenomatous polyposis (FAP). This is an autosomal dominant disease in which patients inherit a mutation in the APC gene located on chromosome 5q21. Such patients develop multiple benign adenomas at an early age at least some of which have a high probability of progressing to carcinoma [15]. To our surprise, the flat, normal appearing mucosa of such individuals showed a marked perturbation in pattern of gene expression compared to the mucosa of low risk individuals. Alterations in gene expression in the high risk, normal appearing tissue amounted to 25% of the sequences assayed, much greater than in the adenomas or carcinomas which subsequently arise [8]. We believe that this marked perturbation indicates that the inherited mutation in the APC gene has pleiotropic effects which initiate many pathways to the transformed phenotype. The relatively lesser extent of alterations in the tumors which eventually arise may be due to the clonal selection of different paths in each neoplastic growth [1].

From the large number of changes detected, we selected 30 clones each of whose mean level of expression in biopsies of FAP high risk flat mucosa differed from the mean level of expression in low risk normal mucosa (Figure 1) [10]. A second screen of the sequences in which the flat mucosa from a different high risk group, hereditary non-polyposis colon cancer (HNPCC), was investigated gave similar results (Figure 1). Indeed, although each of the 30 sequences were expressed at a characteristic higher or lower level in both risk groups as compared to the normal control mucosa, the mean level of expression of each sequence did not differ in comparing the two genetic risk groups [10]. Since HNPCC does not involve inherited mutation at the APC locus, these results suggest that regardless of etiology, risk for development of colonic tumors may be established by similar mechanisms.

PATTERNS OF GENE EXPRESSION

Although the mean level of expression of each of the 30 sequences differed in both the high risk FAP and HNPCC biopsies compared to the low risk normal mucosa, it was clear that there was overlap in the data, and that for each sequence, there were biopsies which were either false positives or false negatives. These data are summarized in Figure 2, in which for each clone, a shaded area indicates that the clone scores the

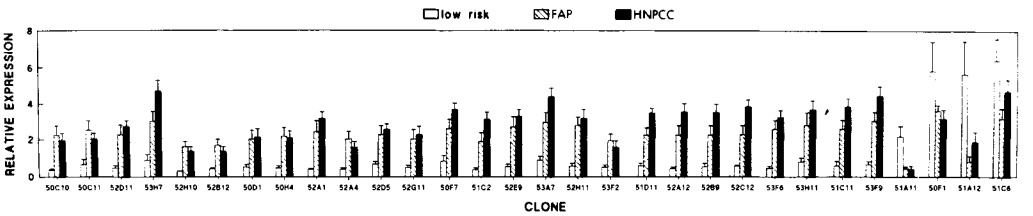

Figure 1. Mean level of expression of selected clones in low-risk mucosa compared to FAP and HNPCC high-risk mucosa [reprinted from reference 10].

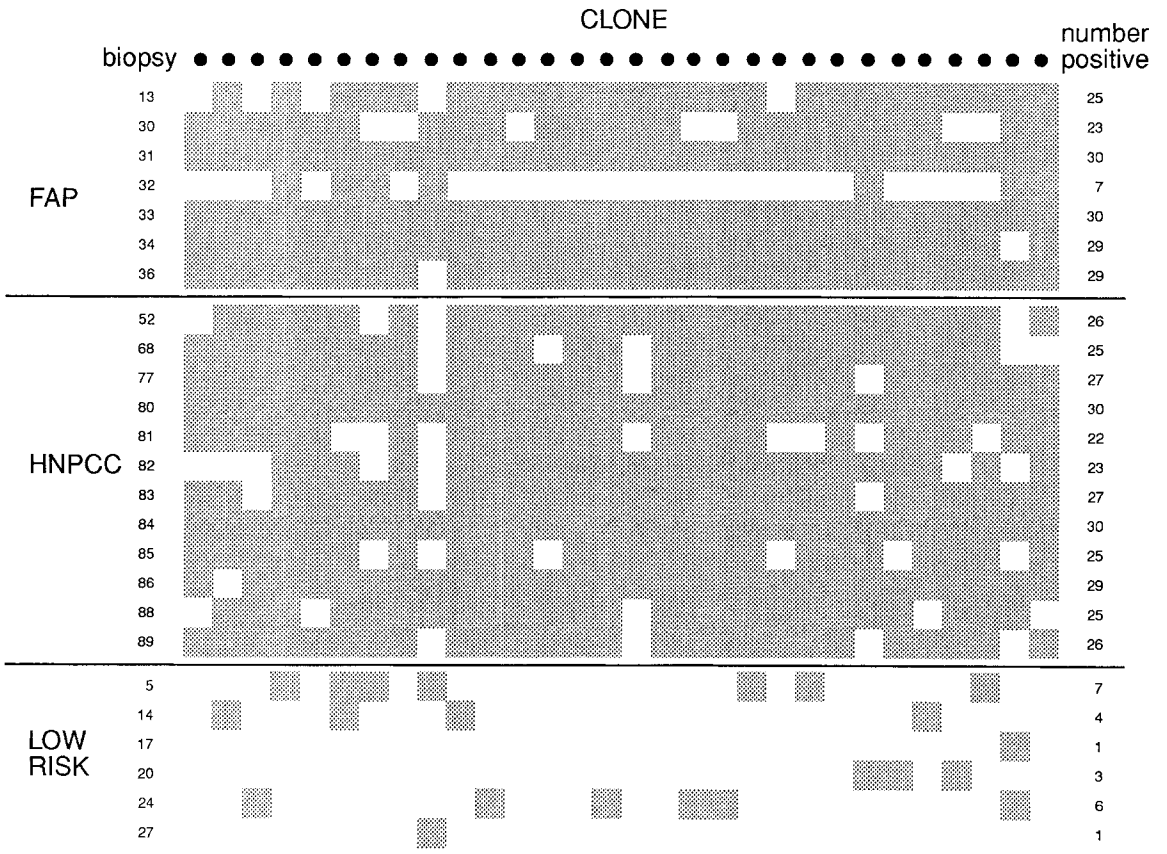

Figure 2. Pattern of expression of selected clones in the low- and high-risk biopsy samples. Each biopsy sample is identified by its number at left; each of the 30 selected clones, in the same order as that shown in Fig. 1, is represented by a solid circle at the top. For each biopsy sample, a shaded area was placed in the clone column if the clone categorized the sample as high-risk. The numbers at right are the number of clones of the 30 selected for which the biopsy sample was so categorized as high-risk [reprinted from reference 10].

biopsy as high risk. Despite the false negatives among the FAP and HNPCC high risk tissue, and the false positives among the low risk normal mucosa, it is clear that the overall pattern of expression can be used to characterize the tissues as high or low risk [10].

HT29 human colonic adenocarcinoma cells can be induced to a more differentiated phenotype by treatment with the short chain fatty acid (SCFA), sodium butyrate (NaBut) [see review in 16]. When the same reference panel of cDNA clones was screened in an identical manner with

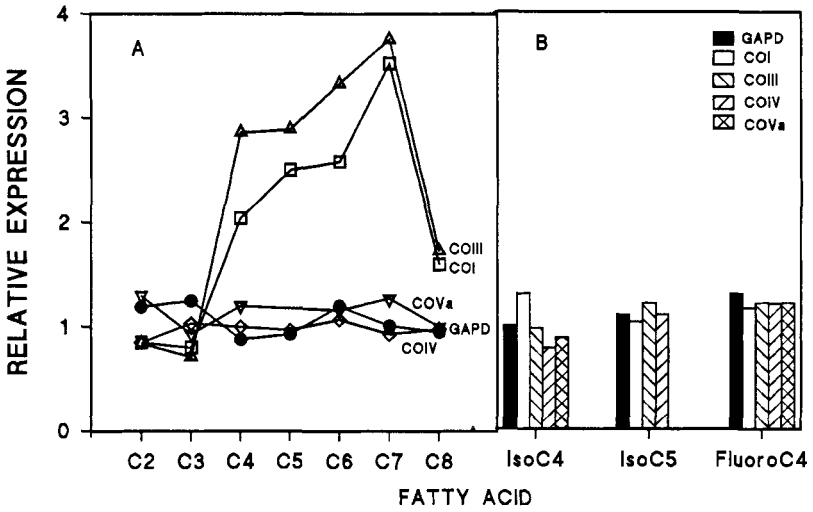

Figure 3. Relative expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPD), mitochondrial genes COXI and COXIII, and nuclear genes COXIV and COXVa. Panel A shows expression in HT29 colon carcinoma cells treated with fatty acids of differing chain length, while panel B shows expression in cells treated with branched C4 and C5 fatty acids, and a fluorinated derivative of C4 [reprinted from reference 17].

probes made from either uninduced or induced HT29 cells, many sequences were found to change in expression. From all the comparisons made, one clone, 50F1, had the following distinctive characteristics. It decreased in level of expression during the progression from normal mucosa to adenoma to carcinoma, and was found at the low carcinoma level in the flat mucosa of FAP and HNPCC patients at high genetic risk for development of colonic tumors. Moreover, upon induction of differentiation of HT29 colonic carcinoma cells with NaBut, the level of expression increased to the levels which characterize the normal mucosa [8]. Sequence analysis determined that this clone was a cDNA of COX III, the third subunit of mitochondrial cytochrome oxidase, which is encoded in the mitochondrial genome [16].

SHORT CHAIN FATTY ACIDS AND MITOCHONDRIAL GENE EXPRESSION

Cytochrome oxidase is a multisubunit mitochondrial enzyme. Some subunits are encoded and synthesized within the mitochondria, and others in the nucleus with subsequent transport of the protein synthesized on cytoplasmic ribosomes into the mitochondria for assembly into a functional enzyme. The detection of alterations in expression of a mitochondrially encoded subunit of this enzyme was significant for several reasons. First, the induction by a short chain fatty acid, NaBut, is of note since SCFAs induce differentiation of colonic epithelial cells both in vitro and in vivo [see discussion in 8,16,17]. Second, the level of SCFAs in the colonic lumen is very high, ranging up to several hundred millimolar [18-20]. Third, the source of these SCFAs is the microbial fermentation of fiber in the gut, and the SCFAs, metabolized directly within the mitochondria, then serve as the principal energy source for colonic epithelial cells [18-21].

We therefore investigated the effects of SCFAs on synthesis of the subunits of COX, and on COX enzymatic activity in purified mitochondria [17]. The results are summarized in Figures 3 and 4. Straight chain fatty acids ranging from 3 to 7 carbons in length are effective inducers of two mitochondrially encoded subunits, COX I and III, while two subunits encoded in the nucleus, COX IV and Va, are unaffected (Figure 3A). Branched SCFAs, isoC4 and isoC5, and a fluorinated non-metabolizable derivative, do not induce expression of any of the subunits investigated (Figure 3B). Figure 4 illustrates that the induction of COX enzymatic activity in mito-

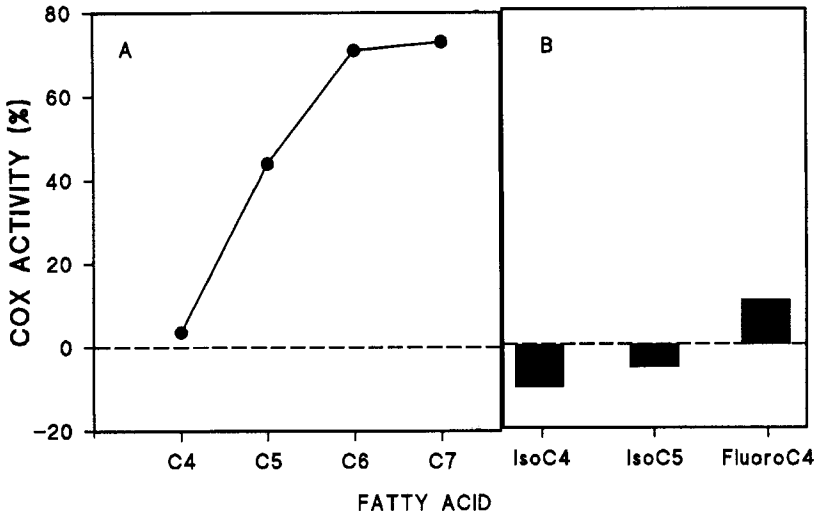

Figure 4. The level of activity of mitochondrial cytochrome oxidase relative to control values in cells treated as in Fig. 3 [plotted from data presented in reference 17].

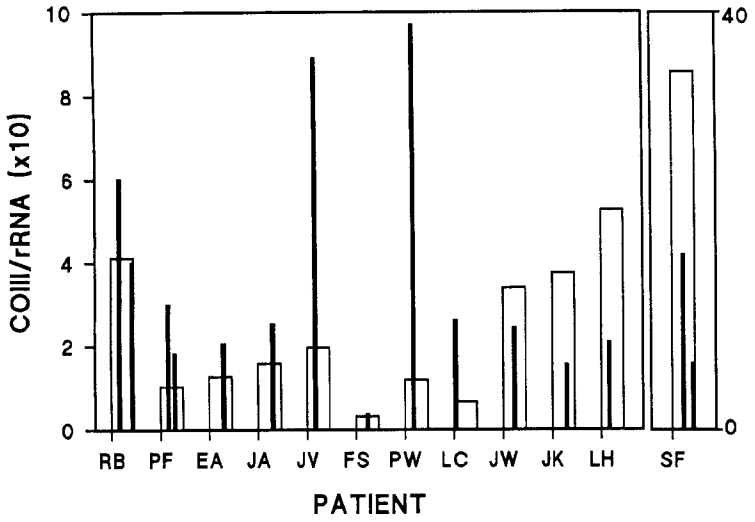

Figure 5. The level of expression of mitochondrial COXIII in the colonic mucosa of high-risk patients both before and following supplementation with 1500 mg/kg $CaCO_3$ per day. The open box for each figure is the expression in a pre-calcium biopsy; the closed boxes are the levels of expression in biopsies taken during calcium supplementation. The closed boxes are positioned within the open boxes to reflect the length of calcium supplementation to that point (i.e., extreme left=0 months; extreme right=7 months). Data are calculated as the level of expression of COXIII relative to rRNA in the RNA isolated from the biopsy.

chondria parallels the effectiveness of the SCFAs in inducing the mitochondrially encoded subunits.

These data lead to the hypothesis that the reduced level of expression of COX III in colonic tumors and tissue at risk is one manifestation of the documented defective structure and function of mitochondria in colonic tumors [22-24]. SCFAs, via their metabolism within the mitochondria, induce the expression of mitochondrial genes and correct this metabolic imbalance, thereby favoring establishment of a more differ-

entiated state. Consistent with the hypothesis that metabolism within the mitochondria is the key event in induction are the data presented in Figures 3 and 4, and the fact that induction of COX III subunit expression returns to baseline unless fresh SCFA is added daily, presumably because the inducer is metabolized over time [17].

This hypothesis leads to directly testable questions: what is the fundamental defect which establishes reduced mitochondrial gene expression in colonic tumor cells and tissue at risk? What are the mechanisms by which SCFAs overcome these blocks to normal levels of expression? Since SCFAs are derived from fermentable fiber, do these results reflect a mechanism by which a component of the diet can modulate risk for development of colonic tumors?

LEVEL OF GENE EXPRESSION AS A MARKER OF RISK

The differential expression of sequences in tissue at risk for development of colorectal cancer (Figure 2) raises the possibility that modulation of such expression could serve as a biomarker in intervention studies to monitor response. We have initiated such a study with Martin Lipkin on HNPCC patients given a daily supplement of 1500 mg/kg $CaCO_3$. Such dietary supplementation is effective in reversing the expansion of the proliferative compartment in the colonic mucosa which is a characteristic in patients at risk for development of colonic tumors [25].

The preliminary results are seen in Figure 5. The pre-calcium level of expression of COX III relative to ribosomal RNA is illustrated by an open box for each patient, with the post-calcium levels indicated by the closed box(es) within the open box. The position of the closed box represents the months following the initiation of dietary supplementation (e.g., 1 month at the left edge of the open box, 7 months at the right edge). For the first 12 patients, there are 7 responders (i.e., patients whose level of COX III expression increases), which is similar to the response rate in terms of the proliferative compartment [26]. The data suggest that response is related to time of treatment. The earlier in the treatment period in which the post-calcium biopsy was taken, the greater seems to be the response. This is particularly evident for patients RB and PF, in which more than one post-calcium biopsy was taken and evaluated. In each case, the earlier biopsy exhibits a higher level of expression than the later one. Also note that the responses of JV and PW, which are very large, are seen in biopsies taken shortly after supplementation begins (both at 3 months), as compared to the lower responses for EA and JA, which were measured in biopsies at 5 months. Thus, there may be an initial response of the mucosa to calcium supplementation, followed by an adaptation.

Clearly, more needs to be understood regarding the kinetics of the response of this mitochondrial gene to calcium and other potential modulators of risk for colonic cancer. Further, as we understand more about the other sequences in the panel which characterize risk (Figures 1 and 2), we may find more useful indices of the effects of various agents.

ACKNOWLEDGMENT

This work was supported in part by Grants CA-41372, CA-40558, and P30-CA-1330 from the National Cancer Institute and a grant from the Aaron Diamond Foundation.

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