

Review

A Functional Genomic Approach for Screening Preneoplastic Stages of Sporadic Colon Cancer

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Abstract. *Colon cancer is a curable disease if detected early. Microarray studies have shown that the malignant phenotype is acquired early on in colon, as in other cancers. Therefore, it becomes important to identify the molecular markers (or genes) whose expressions are implicated in early malignancy, as presented herein. Some of these genes (for example p53, Mch4 and IGFR) were shown to change their expression as they go through the various developmental stages of progression. Thus, studying the expression of these genes through the various stages of colon cancer will enhance our understanding of the mechanisms of carcinogenesis. A functional genomic approach to screen for colon cancer non invasively can be employed to test for several of these genes at the seemingly early stages of crypt foci, thereby facilitating early detection. Individuals showing aberrant expression of these genotypes can then be further screened and followed more thoroughly.*

Colorectal cancer (CRC) is one the leading causes of cancer death in industrialized countries (1). Without preventive intervention, about 5.6% of Americans will develop CRC at some point during their lives (2). When colorectal cancer is diagnosed at an early localized stage, first year survival is 90%; thus, early detection can contribute significantly to the

Abbreviations: ACF, aberrant crypt foci; CRC, colorectal cancer; IHC, immunohistochemical; LCM, laser capture microdissection; RAP-PCR, arbitrary primed PCR; RT-PCR, reverse transcriptase polymerase chain reaction.

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prevention of death from this cancer (3), as shown by the 6-8% overall improvement in colorectal cancer survival during the past 20 years.

The American Cancer Society has established guidelines to screen for colon adenomas in women and men, age 50 and older. The guidelines include fecal occult blood test (FOBT), colon X-ray, flexible sigmoidoscopy, colonoscopy and double contrast barium enema (2). Computer tomography (CT) imaging of the bowl is currently being evaluated (4), as is genetic testing for mutations in DNA present in neoplastic cells excreted in feces (5). Despite these promising avenues of investigation, tests now available neither detect colon cancer in all cases nor are they specific. Furthermore, these tests are often expensive, produce false-positive or false-negative results, and entail discomfort and inconvenience to the patients (6).

The molecular mechanisms and pathways that establish and maintain the balance between cellular proliferation, differentiation and senescence of epithelial cells within the colonic crypt remain largely unknown. Therefore, the identification of genetic profiles in normal colonic crypts might elucidate the complexity of this microenvironment and aid in better understanding of colon carcinogenesis. Moreover, the genetic events leading to the development of CRC are the result of a series of molecular alterations such as mutations, genomic instability, activation of oncogene(s) and inactivation of tumor suppressor genes, in addition to changes in the regulation of genes that play a role in development, differentiation and DNA repair. Other factors such as DNA methylation are likely to contribute to the neoplastic process (7). While the majority of adenomas will never reach malignancy, at present, we cannot accurately predict whether a benign growth will progress because we do not have a clear understanding of mechanisms of tumor progression, especially at the preneoplastic stages of CRC.

Since the first detection of aberrant crypt foci (ACF) in carcinogen-treated mice in 1987 (8), there have been numerous studies focusing on these microscopically visible

lesions both in rodents and humans (9). ACFs have been generally accepted as precancerous lesions with regard to histopathological characteristics, biochemical and immunological interactions, as well as genetic and epigenetic alterations. ACF show variable histological features, ranging from hyperplasia to dysplasia. ACF in human colon are more frequently located in the distal part (left colon) than in the proximal part (right colon), a feature that is also in accordance with more advanced CRC lesions (10).

Cellular proliferation, lineage-specific differentiation, migration and apoptosis are coordinated processes that occur in sequential and spatially organized patterns along the colonic crypt axis. Stem cells at the base of the crypt give rise to progenitor cells, which expand by rapid proliferation along one of three cell lineages (absorptive, goblet or enteroendocrine) as they migrate along the crypt axis toward the lumen surface (11). Although factors that regulate these processes are only partially understood, a number of biochemical events have been implicated, including E-cadherin-mediated cell-cell and integrin-mediated cell-substratum adhesion, chemotactic gradients, extracellular matrix (ECM) and mesenchymal components, cytokines, hormones and growth factors (12). Downstream of these stimuli, signaling pathways and transcription factors, including Tcf-4, MATH-1, the homeobox genes (cdx-1 and cdx-2), kruppel-like factor 4 and several members of the forkhead family of transcription factors were shown to have a role in the coordination of colonic cell maturation (13).

The genetic programming induced by these stimuli to bring about the differentiated phenotype is not well known. Differentiation of colonic epithelial cells also remains a poorly-defined process at the molecular and biochemical levels. At the molecular level, differentiation of absorptive cells begins as a cessation of DNA synthesis, followed by morphological changes such as cellular polarization, and the formation of well-developed tight junctions and microvilli (11, 14). At the biochemical level, increased expression of brush border hydrolases such as alkaline phosphatase, sucrase isomaltase and dipeptidylpeptidase IV, have been described (15). However, other changes are likely to occur as colonic epithelial cells migrate upwards along the crypt axis, including changes in signaling pathways, interactions with the extracellular matrix, metabolic processes, adaptive responses to luminal contents and apoptotic pathways. These changes, however, are only partially characterized.

The immunohistochemical (IHC) expression of carcinoembryonic antigen (CEA), β -catenin, placental cadherin (P-cadherin), epithelial cadherin (E-cadherin), inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and p16^{INK4a} was shown to be altered in rodent and human ACFs (16-20). Moreover, genetic mutations of K-ras, APC and p53, epigenetic alterations of CpG island

methylation, as well as genomic instabilities due to defect in the mismatch postreplication repair system – particularly in dysplastic ACF – have been detected (10).

By studying dysplastic ACFs, it may be possible to learn more about the early development of colon carcinogenesis, as they have been postulated to be precursors of adenoma (9, 10, 21), although it has been suggested that there is an alternative route to colon carcinogenesis, involving microsatellite instability due to defect in mismatch DNA repair, that does not proceed from the classical multistage process of adenoma to carcinoma sequence progression (7, 12).

Functional Genomics in Colon Cancer Diagnosis

Functional genomics, or transcriptomics, refer to the study of the direct expression of gene products [*i.e.*, conversion of messenger (m) RNA into a copy (c) DNA]. The expression of individual genes may be altered either by mutations in the DNA, or by a change in their regulation at the RNA or protein levels. Working with DNA has been relatively much easier than working with the easily degradable mRNA (23). However, mutation detection in the DNA of oncogenes and suppressor genes suffers from: a) the existence of these genes in fewer than half of large adenomas and carcinomas, b) the presence of the markers in non-neoplastic tissues, c) mutations found only in a portion of the tumor, and d) mutations often produce changes in the expression of many other genes (24). On the other hand, protein-based methods are not suited for screening and early diagnosis because proteins are not specific to one tumor or tissue type (*e.g.*, CEA), and because detection of these markers in blood often signifies the presence of an advanced tumor stage (25). Furthermore, the human proteome is complex (ranging from 100,000 to several million different protein molecules) and more complications arise because no function is known for more than 75% of predicted proteins of multicellular organisms. Additionally, the dynamic range of protein expression is as large as 10^7 (26). Tissue microarray studies revealed that protein expression vastly exceeds RNA levels, and only phosphorylated proteins are involved in signal transduction pathways leading to tumorigenesis (27). Moreover, many of the phenotypic variations among organisms are related to changes in gene expression and not to alterations in protein sequences (28). The defining factor that distinguishes the many phenotypic cell lineages that develop from fertilized eggs are the gene expression profiles of the cell lineage as they develop. The specific components of the developing organism that determine which genes are expressed or not are the regulators of gene expression, known as transcription factors (29). The diversity of gene expression arises, to a large extent, by alternative RNA splicing, which has alleviated concerns that arose following the recognition that the human genome contained only

about 30,000 genes. Alterations in splicing events can thus lead to human disease, such as cancer (30). Hence, an RNA expression marker is preferable to either a DNA- or a protein-based marker, especially since this messenger molecule can now be stabilized by commercial kits so it does not readily fragment, making it amenable to handling and processing (25, 28).

There have been two general new approaches to studying gene expression: (a) the open system approach utilizing techniques such as differential display and serial analysis of gene expression technologies, and (b) the closed system approach utilizing several forms of high-density cDNA arrays or oligonucleotide chips and RT-PCR methodologies. While closed systems are suitable for the initial screening of known sequences, the open systems are advantageous in evaluating the expression patterns of unidentified genes (6). Many of the expression techniques were limited by the requirement of a large amount (up to 5 µg) of non-fragmented DNA-free mRNA. However, differential screening of cDNA arrays can be improved upon by systematically increasing the concentration of rare transcripts used to screen such arrays, as, for example, by amplifying the messages *via* RNA arbitrary primed PCR (RAP-PCR) fingerprinting (31). Advances in cDNA microarray technology enabled the delineation of global changes in gene expression.

The development of colon tumorigenesis requires mutations of multiple key genes, which, through both direct and indirect mechanisms and clonal selection, result in alternating expression of a myriad of genes (32, 33). Multiple studies have demonstrated the ability of microarrays for classification and prognosis of multiple cancers, including colon tumors, either in a supervised or in an unsupervised manner (34, 35). While the gene list generated from these and similar studies are of biological interest, for the most part, their predictive power has not been tested either internally (*i.e.*, by a cross validation statistical strategy) or externally (*i.e.*, by using an independent sample test) (36). In addition, although there have been several studies that showed the potential use and power of gene expression profiling for the classification of cancer, there have been fewer reports in which gene expression profiling has been successfully applied to identifying key genes critical for tumorigenesis, because the techniques vary greatly in their sensitivity and specificity and the ease of their performance (6). Moreover, microarray studies, which are not standardized, need to be confirmed by other expression techniques before any conclusions from them are reached (6). Identification of critical genes is of great importance since these genes are targets for the development of tumor-specific drugs with fewer toxic side-effects; in addition, studying these genes allows for better understanding of tumor biology (38).

Gene Expression Patterns in Colon Crypts and those that Define Colon Cell Maturation

In this article, four *in vitro* and *in vivo* studies are reviewed that employed microarrays for elucidating patterns of gene expression in colon crypts and those that defined colon cell maturation in both human and animal models (39-42).

Purification of cells from heterogeneous tissue and production of RAP-PCR probes. The study of gene expression in heterogeneous tissue samples was facilitated by the advent of a relatively new purification technique that separates target cells from surrounding stroma and inflammatory cells. This technique, known as laser capture microdissection [LCM] (43), eliminated the uncertainties associated with older manual, ablation or mechanical microdissection techniques (23). An alternative approach has been to mathematically subtract non-tumor cell-derived signatures from the gene expression data "*in silico*", including stromal and muscle cell-specific genes, and genes related to immune function that are expressed in lymphocytes (44, 45). While these modifications have improved the discrimination power, LCM represents the "gold standard" of signal enhancement (36). It has been argued that analysis of tumor cells in the context of its host environment may be more informative than analysis of pure cell populations (46, 47). It is not possible to ascertain which approach is more informative until a definitive study, that compares the use of whole tissue *versus* microdissected tumor, is conducted (36).

To circumvent the issue of tissue heterogeneity, a new technology, called "transcriptional imaging", has been developed. In this method, a cocktail of probes directed against different target genes is generated by labelling with different fluorochromes. Addition of these probes to a heterogeneous tissue samples results in hybridization of several spectrally distinct probes to their respective target mRNAs at specific sites at which target genes are transcribed (*i.e.*, transcription sites) (48, 49). This approach could theoretically identify subgroups of cells with distinct phenotypes that may represent a minor percentage of the overall cell population. This sensitivity may not be achieved through microdissection of individual cells coupled with gene expression profiling because such cells cannot be identified *a priori*, and, therefore, the number of individual cells to be microdissected and analyzed would be quite large (36). Other advances in RNA amplification have been developed in order to reduce the amount of RNA required for analysis (50, 51).

For producing improved probes that can screen microarrays, a modification of the original RAP-PCR protocol (37), termed "nested" RAP PCR, was employed (41, 42). Extracted RNA was reverse transcribed for first

Table I. Differentially expressed genes in basal versus luminal part of normal colonic crypt, and in low-grade dysplasia (D1) versus normal colonic human tissue samples.

Gene	Full Name	Average Regulation	GenBank Accession #
c-jun	c-jun proto-oncogene	Up 40-fold in lumen	J04111
Mch4	Apoptotic protease Caspase 10	Up 12-fold in lumen	U60519
P58/HHR23B	XPC group C repair complementing protein	Up 36.5-fold in basal part	D21070
E2f1	pRb-binding protein RBBP3, RBAP1, PBR3	Up 17.5-fold in basal part	M96577
p21-rac1	ras-related protein TC25	Up 3.3-fold in D1*	M29870/M31467
MAPKa38	Mitogen activated protein kinase 38	Up 3.2-fold in D1+	L35253/L35263
IFGR	Interferon gamma receptor	Up 3.5-fold in D1+	A09781
TSP2	Thrombospondin 2	Down 8.9-fold in D1+	L12350
FAST	Fas-associated serine/threonine kinase	Down 5.7-fold in D1+	X86779
P53	p53 cellular tumor antigen	Down 11.3-fold in D1+	M14694/M14695

*In 4 out of 6 colon cancer patients.

+In 3 out of 6 colon cancer patients.

Modified from references 41 and 42.

strand synthesis using a 10-mer arbitrary primer to produce a single-stranded cDNA. Then, PCR for production of the second strand was performed using a nested strategy that employed a pre-amplification step using a 10-mer arbitrary primer 5'- ACCAGGGGCA -3', followed by a second amplification step with a nested 10-mer arbitrary primer 5'-CCAGGGGCAC -3', which had one additional nucleotide at the 3'-end of the first arbitrary primer sequence. Confirmation of differential expression on microarrays for certain genes was carried out using both RT-PCR and IHC methods. Non-isotopic labelling methods can also be employed, which obviate the hazards and inconvenience associated with the use of radioactive material in clinical settings (23).

To deal with the issue of standardization, a number of consortia have been formed and new systems implemented: for example, the recommended use of universal RNA pool for platforms that utilize a two-color hybridization system in cDNA microarrays (52) and compliance with MIAME standards (53). Other advances in profiling methods are now available, such as BAC arrays for screening the whole genomic DNA for genome imbalances such as insertions and deletions (e.g., comparative genome hybridization, CGH, arrays) (54). Moreover, by hybridizing DNA to the same cDNA or oligonucleotide microarray, it is possible to obtain information on DNA copy number, which can then be correlated with variation in gene expression measurements (55). Moreover, array-based technology can be used to study epigenetic modifications such as DNA methylation using CpG island arrays (56), or changes in protein (26), which will allow for studying changes not evident by gene expression profiling. This "system biology" approach could ultimately produce a more comprehensive method to analyze a complex phenomenon such as the multistep process of development and progression of colon cancer.

The study of normal cell maturation has been enhanced by using *in vitro* cell culture models such as Caco2 cell lines. On contact inhibition, these cells are programmed to undergo cell cycle arrest with cells accumulating in the G₀/G₁-phase of the cell cycle, followed by a cascade of events leading to differentiation along the absorptive cell lineage, which are triggered by certain mechanisms (57). In a microarray analysis of 17,280 sequences during spontaneous Caco2 differentiation (39), 13.2% of all genes were altered in expression. The majority of the changes (70%) were reduction in expression of genes involved in cell cycle regulation and nucleic acid synthesis, RNA processing, translation, protein folding and degradation; a finding consistent with cell differentiation reflecting increased cellular specialization. On the other hand, genes involved in xenobiotic and drug modification, extracellular matrix deposition and lipid metabolism were up-regulated. One likely trigger is the β -catenin-TCF signaling pathway. The increase in ECM-associated genes suggests increased deposition for ECM may be a function of differentiated colonic epithelial cells, such as enhanced TGF- β signaling. The increased expression of xenobiotic and drug detoxification genes may reflect an important defense mechanism that colon cells have developed to manage their increased exposure to luminal compounds as they migrate up the crypt axis. However, in the context of a tumor, increased expression of these genes may lead to increased resistance to specific chemotherapeutic agents (39).

A general up-regulation of genes involved in lipid metabolism and transport probably reflects a role for differentiated enterocytes in the absorption and metabolism of luminal lipids and their packaging into chylomicrons for transport to the liver. The analysis also revealed a number of pathways that may drive these reprogramming events, including chromatin-modifying enzymes, other signaling

pathways such as MAPK and RXR, as well as other transcription factors. Thus, this study demonstrated that the genetic programming that accompanies colon cell maturation occurs in a highly organized and coordinated manner. Moreover, the complexity of change, even within some functional groups of genes and along a single differentiation lineage, emphasizes that cell maturation reflects an interplay and balance among many pathways (39).

In a study that employed a mouse model of sporadic colon cancer in an attempt to develop a molecular signature to determine whether cancer risk is related to histopathological features of preneoplastic ACF, gene expression analysis was performed on ACF from two mouse strains with differing tumor sensitivity to the colonotropic carcinogen azoxymethane, which produces tumors and preneoplastic lesions in mice that closely resemble key molecular features of human colon cancer. ACF from sensitive A/J mice were considered at high risk, whereas ACF from resistant AKR/J mice were considered at low risk for tumorigenesis. IHC analysis of the proliferating cell nuclear antigen, p53 and several components of the Wnt signaling pathway, including β -catenin, APC, cyclin D1 and c-Myc, afforded minimal discrimination of ACF (58-60). This limited ACF discrimination prompted a genome-wide array-based expression analysis to identify discriminatory molecular profiles. A/J and AKR/J mice received weekly injections of azoxymethane (10 mg/kg body weight), and frozen colon sections were prepared 6 weeks later. IHC was performed using biomarkers associated with colon cancer, including adenomatous polyposis coli, β -catenin, p53, c-myc, cyclin D1 and proliferating cell nuclear antigen. Hyperplastic ACF, dysplastic ACF, microadenomas, adjacent normal-appearing epithelium and vehicle-treated colons were laser captured and DNA was linearly amplified (LCM-LA) and subjected to cDNA microarray-based expression analysis. Patterns of gene expression were identified using adaptive centroid algorithm. These analyses enabled a correlation of transcriptional profile(s) within lesion subtypes with respect to morphology stage, an approach that increases understanding of the transcriptional programs that influence tumor prognosis or growth arrest of putative precancerous lesions. ACF from low- and high-risk colons were not discriminated by IHC, with the exception of membrane staining of β -catenin. To develop genetic signatures that predict cancer risk, LCM-LA RNA from ACF was hybridized to cDNA arrays. Of 4896 interrogated genes, 220 clustered into 6 broad clusters. A total of 226 and 202 genes were consistently altered in lesions from A/J and AKR/J mice, respectively. Although many alterations were common to both strains, expression profiles stratified high- and low-risk lesions, and the genes that were differentially expressed in A/J and AKR/J ACF represent only a small percentage (<5%) of the total

number of genes interrogated, underscoring the fundamental similarity of lesions at the earliest stages of tumorigenesis. For example, genes that are elevated in the A/J dysplastic ACF and down-regulated in the AKR/J dysplastic ACF include ASML3a, MAP-17, Rab24, SPII, thrombospondin 4 and Grim-19. Cluster analysis identified a gene panel that was significantly induced in resistant AKR/J ACF. These genes include IEX-1, Mfge8, Rnh1, Psmd13 and Usfs2. The increased expression of a subset of genes involved in protein biosynthesis, DNA repair, transcription regulation, members of the nuclear factor- κ B family, ion transport and cell metabolism raise the possibility of establishing a gene signature for low-risk ACF. Each of the genes represented within this cluster have putative functions that could potentially play a role in limiting ACF progression. These data demonstrate that ACF with distinct tumorigenic potential have distinguishing molecular features, that provide insight into colon cancer promotion, in addition to identifying potential biomarkers for determining colon cancer risk in humans (40).

Table I shows the differentially expressed genes in the basal or luminal part of normal colonic crypts in 2 independent measurements, and in low-grade dysplasia of colon cancer patients found in at least 3 out of 6 patients studied (41, 42). The importance of 10 of these genes for colon neoplasia is discussed below.

c-jun proto-oncogene

The jun proto-oncogene, mapped to chromosomal region 1p31-32, encodes a protein that is functionally and biochemically identical to the transcription activator protein 1 (AP-1) factor. Jun is an intronless gene that yields several transcripts due to 5' and 3' heterogeneities. The gene is functional since transfection experiments showed that it encodes a trans-acting factor that stimulates transcription of the AP-1-dependent reporter gene (61).

Activation of c-jun-N-terminal kinase (JNK) by various chemotherapeutic agents in human carcinoma cells was shown to be crucial for the induction of cell cycle arrest and apoptosis (62). On their way to apoptosis, murine Balb/c-3TC cells showed up-regulation of c-jun, c-fos, c-myc, cdc2 and Rb phosphorylation, resembling events of early cell-cycle traverse by expressing early G1 genes (63). Moreover, up-regulation of c-jun, c-fos and cc-2 gene expression was observed in mucosa adjacent to colon carcinoma (≤ 4 cm) in tissue samples obtained from colon adenocarcinoma patients (64). A RAP-PCR probe for screening a cDNA microarray in LCM of the luminal part of the colon crypt showed a 40-fold increase in the expression of the c-jun proto-oncogene (41). Thus, it is important to look at the degree of expression of this and other highly expressed genes in colon lumen and in cells isolated from the stool of

CRC patients, as these cells in the lumen are periodically shed as colonocytes in human excrement and can be easily isolated and studied non invasively (28).

Apoptotic protein Mch4

Mch4 or caspase 10/a is a member of the aspartate-specific cysteine proteases (ASCPs), renamed the caspases (for cysteine-aspartic acid proteases), which are implicated as mediators of apoptotic cell death. Caspases are present as inactive pro-enzymes comprising a prodomain and a catalytic protease domain that can be further processed to give a large and a small subunit. Subsequent activation of caspases lead to a signaling cascade of events, with caspase 10 at or near the apex of an apoptotic signaling pathway associated with the death receptor Fas and tumor necrosis factor (TNF) receptor 1 (65). The putative active site of Mch4 contains N-terminal FADD-like death effector domains, suggesting a role of this protein in the Fas-apoptotic pathway. Granzyme B cleaves proMch4 at a homologous IXXD processing sequence to produce mature Mch4, alluding to the notion that the mature protease targets other caspases such as CPP32 and Mch3 in the apoptotic cell (66). The expression of Mch4 was up-regulated 12-fold in the luminal part of normal colonic crypt cells (isolated by LCM) ready to be shed in the lumen, using PAR-PCR purified probes to screen a cDNA expressing microarray (41). On the other hand, another study, examining differential gene expression in LCM CRC tissue by a cDNA microarray, showed Mch4 to be down-regulated in patients with advanced stages of CRC who underwent colectomy (67).

Xeroderma pigmentosum group C repair complementing protein p58/HHR23B

The xeroderma pigmentosum group C (XPC) protein, specifically involved in genome-wide damage recognition for nucleotide excision repair (NER), was purified from HeLa cells in a cell-free system as a tight complex with one of two human homologs of RAD23 NER protein, designated HHR23B (human homolog of Rad 23) in the budding yeast *Saccharomyces cerevisiae*, having a molecular mass of 58 kDa (68). This XPC-HR23B complex, which has a high affinity for ss- and ds-DNA, and functions as initiator of the NER reaction, can recognize many lesions, including UV-induced (6-4) photoproducts, N-acetyl-2-aminofluorene adducts and an artificial cholesterol moiety. It can also function as a damage detector to initiate NER reactions (69). On the other hand, HR23B alone does not have affinity for DNA (41). The XPC-HR23B complex binding to a lesion after damage to DNA leads to recruitment of the transcription factor IIIH (TFIIH) involved in damage verification and DNA unwinding around damage through

binding of that factor to the carboxy terminus 125 amino acids of XPC (70), and further recruitment of other repair factors such as XPA, XPG and replication protein A (71).

A gene expression strategy, using a differential display approach of generating differential screening probes with increased representation of rare mRNA to screen cDNA microarray, found p58 to be up-regulated on average 36.5-fold in the basal part of colonic crypts (41). No other studies on the expression of this repair gene in advanced cases of colon cancer have been reported.

PRB-binding protein E2f1

Studies have shown that the activity of the transcription factor E2f1 is inhibited by direct binding with the retinoblastoma protein pRb, leading to loss of a functional pRb and progression to neoplasia (72). On E2f1, a pRb binding site of 18 amino acids has been identified within the C-terminal transactivation domain. Transactivation by E2f1 involves binding to DNA, and the reversal of binding upon binding of the pRb may turn E2f1 into a repressor (73). Phosphorylation of E2f1 on serine 375 enhances pRb binding *in vitro* (74). Induced expression of E2f1 stimulates mRNA NPTA gene expression, which is a substrate of cyclin E-Cdk2 kinase believed to play a critical role in coordinating transcriptional activation of histone genes during the G1→S-phase transition and in S-phase entry in the cell cycle (75). The expression of E2f1 was up-regulated 17.5-fold in the basal part of normal colon crypt cells using PAR-PCR enriched probes in a cDNA expression array (41). No other studies on the expression of this transcription factor in advanced cases of CRC have been reported.

P21-rac1 (ras-related protein TC25)

A family of ras-related, membrane-associated, GTP-binding proteins, designated rac1 (ras-related c3 botulinum toxin substrate), has been isolated from a differentiated HL-60 library. It encodes a protein that is 92% homologous and shares ~30% amino acids with the human rho group of ras-related genes. Nucleotide sequence analysis predicted rac 1 protein to contain 192 amino acids with a molecular mass of 21,450 Daltons, and to possess 4 of the 5 conserved functional domains in ras associated with binding and hydrolysis of guanine nucleotides. The rac1 also contains the COOH-terminal consensus sequence Cys-X-X-X-COOH, which localizes ras to the inner plasma membrane and the residues Gly¹² and Ala⁵⁹, at which sites mutations elicit transforming potential to ras. Transfection experiments demonstrated that rac 1 is a substrate for ADP-ribosylation by the C3 component of botulin toxin (76).

A mixed-oligonucleotide probe corresponding to the conserved ras domain Asp-Thr-Ala-Gly-Gln-Glu (residues

57 to 62), used to screen a human teratocarcinoma (TC) cDNA library derived from undifferentiated NTera2 cells, identified a nucleotide sequence within a 832-base per a cDNA insert identified as TC25/rac1 (77). Rac-1 protein is involved in several transduction pathways that regulate NF κ B-dependent transcription and MAPKK-1/ERK-2 pathways (78, 79). In a study looking at gene expression of colon adenomatous crypt cells purified by LCM, and using nested RNA RAP-PCR to isolate probes for testing a cDNA expression microarray, p21-rac1 was found to be up-regulated 2.3-fold in 4 out of 6 CRC patients (32). IHC studies, but not RNA expression by Northern blot in colon adenocarcinoma tissue, showed p21 to be up-regulated in mucinous colon cells, suggesting that these phenotypes may have developed along different pathways (80).

MAPK p38 α

Originally described as a 38-kDa polypeptide that underwent Tyr phosphorylation in response to endotoxin treatment and osmotic shock, the p38 α isoform was purified by anti-phosphotyrosine immunoaffinity chromatography (81). cDNA cloning revealed that p38 was the mammalian mitogen activated protein kinase (MAPK) homolog most closely related to the HOG1 gene, the osmosensing MAPK of the yeast *S. cerevisiae*, and, like HOG1, contains the phosphoacceptor sequence Thr-Gly-Tyr (82). The amino acid sequence of human p38 was 99.4% identical to mouse p38 and, also like the murine p38, the dual phosphorylation site of human p38 was characterized by a TGY sequence. This highly conserved nature of p38 suggests that it has a role in regulating cellular responses. MAPK p38 has been involved in various signal transduction pathways regulating cyclin D1 and tristetraprotein (a regulator of TNF α mRNA stability) (83, 84).

An alternate activation and autophosphorylation non-enzymatic mechanism for MAPK p38 α , that depended on interaction of p38 α with TAB1 [transforming growth factor (TGF)- β activated protein kinase 1 (TAK1)-binding protein 1], was found. The TAB1-p38 α pathway complexes with these TNF receptor-associated factor 6 (TRAF6). This alternate autoactivation mechanism could be an important activation pathway operating in parallel with kinase cascades to regulate intracellular signaling (85). The MAPK p38 α activation – BAX expression pathway may also be involved in apoptosis induced by oxidative stress (86). A gene expression pattern of LCM-colonic adenoma crypt cells with low-grade dysplasia showed MAPK p38 α to be up-regulated 3.2-fold in 3 out of 6 CRC patients (42).

Interferon gamma receptor (IFGR)

The pro-inflammatory cytokine γ is a homodimeric glycoprotein with pleiotropic immunologic functions, primarily secreted by activated T cells and natural killer cells.

IFGR has many immunological functions, controls cellular proliferation, and is involved in tissue-mediated damage and apoptosis (87). Two components of the surface receptor, the α chain (also known as INF γ R, or INF γ R1 or CD119) and the β chain (or INF γ R2), have been discovered. Specific residues within the cytoplasmic domains of both the α and β chains of the INF γ R are critical for transducing the INF γ signal from the cell surface to the nucleus through the activation of intracellular signaling cascades, ultimately leading to regulation of gene expression (88).

Studies have shown that INF γ exhibited either a suppressing or enhancing effect on metabolism of several tumor cells, including human adenocarcinoma cells, depending upon the time of treatment of cells or to the properties of the cell line used (89). Inducible nitric oxide synthase (iNOS) mRNA occurred within 2 hours in differentiated villous enterocytes of Caco2 colon adenocarcinoma cells following treatment with INF γ , attributable to an increase in the number of INF γ receptors, and the transcription factor NF κ B is believed to have a role in the process (90).

In a study looking at gene expression profiles of LCM colonic crypts of adenoma patients with a low-grade dysplasia using nested RAP-PCR probes in a cDNA microarray expression method, INF γ was up-regulated 3.5-fold in 3 out of 6 patients (32). On the other hand, in another expression study in LCM colon tissue using oligonucleotide array, INF γ was shown to be 4-fold down-regulated in a more advanced stage of colon adenocarcinoma as compared to normal colon tissue (91).

Thrombospondin 2 (TSP2)

The thrombospondin (TSP) family of proteins consists of at least 5 related extracellular matrix glycoproteins encoded by distinct genes. ALL TSP proteins possess similar structural modules including epidermal growth factor-like type 2 and a calcium sensitive disulfide-bonded trimer with a molecular mass of approximately 145 kDa (92). Human TSP2 was discovered during the screening of a human fibroblast cDNA library as the second member of the TSP gene family. TSP2 is a 420-kDa glycoprotein, and TSP2 and TSP1 share 2 additional modules such as a procollagen homology region and 3 properdin-like type 1 repeats (TSRs). Despite the high degree of similarity in the coding regions of TSP1 and TSP2, the DNA sequences of the 2 promoters are very different, leading to different biological responses and functions between TSP2 and TSP1 (93). High levels of human TSP2 mRNA expression were reported in aortic, cardiac, muscular, fetal, endocrine, immune and nervous tissue (94). TSP2 gene expression is significantly correlated with decreased vascularity in various tumors, including colon cancer (95), was shown to inhibit angiogenesis (64) and tumor growth in both humans and mice (96), and suppressed proliferation of endothelial cells (97).

The role of overexpression of the TSP2 gene and its effect on other genes thought to be involved in the breakdown of the extracellular matrix was investigated in a cDNA microarray study in the colon carcinoma cell line SW480 that was transfected with the TSP2 gene. The transformants with the human TSP2 gene overexpression showed a down-regulation of matrix metalloproteinase 2 (MMP2) and MMP9 in comparison to those with the vector control. Protein production of MMP2 and MMP9 decreased in the transformants overexpressing the TSP2 gene. Conversely, the SW480 transformants showed up-regulation of MMP12 and MMP17. These results suggested that the TSP2 gene is a multifunctional modulator of remodeling tissue in which matrix degradation is required for cancer progression (98).

In colon cancer patients, the expression of the immunosuppressive cytokine interleukin 10 (IL-10), produced by T lymphocytes, was found to be correlated with TSP1 and TSP2 gene expression and resulted in decreased venous vascular involvement in colon cancer (99). A cDNA microarray study showed TSP2 to be 8.9-fold down-regulated in low-grade dysplasia of the colon (42). No other studies on the expression of this gene in advanced cases of colon cancer have been reported.

FAST kinase

FAST is a serine/threonine kinase that binds to the carboxyl terminal of the RNA recognition motif (RRM)-type binding protein TIA-1, that has been implicated as an effector of apoptosis in susceptible lymphocytes causing the phosphorylation of TIA-1. Phosphorylation of this binding protein precedes the onset of DNA fragmentation suggesting a role in signaling downstream events in the Fas-mediated apoptotic transduction pathway (100). Microarray studies showed that FAST kinase is overexpressed in the peripheral blood of atopic asthmatic and non-asthmatic patients (101). In a cDNA microarray study carried out on liver cells expressing the transcription factor hepatitis B viral X protein (HBx), the cells showed elevated expression of the apoptotic genes FAST kinase and BAK, among others, illustrating the selective transcriptional regulation by HBx in the human liver cells (102). A cDNA microarray study showed that FAST kinase was 5 to 7-fold down-regulated in low-grade dysplasia of the colon in 3 out of 6 CRC patients (42). More work is needed on the role of the expression of this kinase in CRC.

P53 cellular tumor antigen

The tumor suppressor gene p53 is a key regulator of cell growth and apoptosis, and the gene is found to be mutated in about 50% of human malignancies. P53 acts as a negative regulator of transcription, resulting in suppressing cell

proliferation and induction of apoptosis. The expression of p53 in CRC has been reviewed earlier, and in most cases its expression was found to be up-regulated (7, 103-105). However, these studies were carried out on patients with advanced colon cancer. In one study that looked at the expression of genes in preneoplastic lesion of the colon by a cDNA microarray, the p53 cellular tumor antigen was found to be down-regulated 11.3-fold in 3 of 6 patients (42). It seems that some of the genes, such as p53, change their expression as they progress through the various stages of CRC carcinogenesis.

P53 is a tetramer. A defect in one of the subunits will have a profound effect on its overall function. Although p53 has been dubbed the guardian of the genome, it is mutated in only 50% of human cancers. This leaves the other 50% of human cancers as having normal homozygous p53. So, in multistage carcinogenesis involving defects/mutations of genes other than p53, it is possible that p53, which has a very short half-life, may be highly expressed in the early stages of the disease. The working model envisioned for p53 expression in some cancer cells is that, in these early stages of the disease, an inherent genomic instability induces the expression of p53. This, in turn, induces cell cycle arrest at G1 and the onset of repair when possible. If repair is impossible, it will induce apoptosis of the damaged cells. If the progression of the disease is uncontrolled, a similar rate of p53 expression cannot be sustained since its negative regulator, MDM2, will be activated to ubiquitinate p53. Over the long haul, p53's function will be compromised. This hypothesis may explain the relatively slow progression of the neoplasia over time, because cell growth is tempered by a high rate of p53-induced apoptosis. However, as the disease progresses, high levels of p53 secretion result in profound defects in non-responsive cell function. These functional defects, combined with abnormal cell proliferation, lead to full-fledged cancer. As the cancer cells increase in number, they induce the down-regulation of such genes as JUN, ATM (up-regulator of p53), thereby liberating the cells from the pro-apoptotic effects of p53 and resulting in an increased tumor cell burden. Although the loss of ATM and other inducers of p53 may be gradual in the early stages of disease progression, it is likely that the p53 cancer network will be dysfunctional enough to stagnate the feedback loop for p53 expression. Hence, the role of p53 is, in many ways, a paradox (*i.e.* secreted p53 may induce apoptosis and block cell growth/differentiation, but it may also facilitate repair). This duality of function can create a microenvironment primed for disease progression. In addition to the above-mentioned pathways for p53, it should be noted that, when considering mechanisms underlying the alteration of p53 expression, we also have to take into account the various viral oncogenes (*e.g.*, CMV) that ultimately bind to p53 and alter its regulatory properties. Mapping out each and every

upstream and downstream gene involved in the p53 pathway may allow us to understand why its expression changes during the course of cancer development (7, 39, 104, 106).

Conclusion

Expression profiling of preneoplastic stages of colon cancer showed that the E2f1, p58/HHR23B, Mch4 and c-jun genes have roles in the normal physiologic processes of normal colonic crypts to maintain the balance between proliferation and apoptosis. The genes p58/HHR23B and Mch4 might be particularly interesting candidate genes for molecular mechanisms responsible for malignant transformation in colonic crypts (41). Genes expressed in the luminal part of the colon have particular diagnostic relevance, as they might be studied in a non invasive manner in colonocytes shed in and passed with the human stool (6, 28, 104). In adenoma with low-grade dysplasia, up-regulation of proliferation-associated genes (p21-rac1, MAPKa38 and IFGR) and down-regulation of apoptotic-related genes (TSP2, FAST kinase and p53) (42), and the urokinase receptor gene (106) were observed, indicating that molecular changes leading to transformation have already started in these precancerous sites.

Microarray studies showed that ACF, believed to be the earliest observed histopathological stage in colon carcinogenesis (10), has already started the steps that will eventually lead to metastasis (107). Thus, a molecular approach to screen for the above reviewed genes, although none of them has been employed in clinical decision making in sporadic colon cancer, would be to extract RNA from colonocytes in the stool of these preneoplastic patients, reverse transcribe it and test for the aberrant expression of these genes by a reliable functional genomic assay such as real-time RT-PCR. An aberrant expression of a few of these genes would indicate that such individuals might be at risk of developing colon cancer, thus requiring frequent colonoscopic examinations at regular intervals looking for polyps that can be tested for further abnormalities. There is a need to perform clinical studies to validate the importance of functional genomics in developing markers to screen for colon cancer, particularly at the early stages of the progression of this disease.

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