Genetic signatures of High- and Low-Risk Aberrant Crypt Foci in a Mouse Model of Sporadic Colon Cancer

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ABSTRACT

To determine whether cancer risk is related to histopathological features of preneoplastic aberrant crypt foci (ACF), gene expression analysis was performed on ACF from two mouse strains with differing tumor sensitivity to the colonotropic carcinogen, azoxymethane. 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. 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. Immunohistochemistry 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 RNA was linearly amplified (LCM-LA) and subjected to cDNA microarray-based expression analysis. Patterns of gene expression were identified using adaptive centroid algorithm. ACF from low- and high-risk colons were not discriminated by immunohistochemistry, 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 six broad clusters. A total of 226 and 202 genes was 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. These data demonstrate that ACF with distinct tumorigenic potential have distinguishing molecular features. In addition to providing insight into colon cancer promotion, our data identify potential biomarkers for determining colon cancer risk in humans.

INTRODUCTION

The majority of colorectal cancer cases are sporadic, characterized by pathologically defined stages, including formation of preneoplastic aberrant crypt foci (ACF), preinvasive adenomas, and malignant carcinomas (1–3). ACF, although not yet fully committed to neoplasia, are stratified into two broad classes, hyperplastic and dysplastic, based on morphology and predicted biological behavior (4, 5). Histomorphologic grading of ACF, in terms of size and dysplasia, represent one of the few biomarkers of colorectal cancer risk (6–10). Therefore, establishing molecular criteria that enhance prediction of ACF progression is relevant to clinical prognostication.

The colonotropic carcinogenic, azoxymethane, produces tumors and preneoplastic lesions in mice that closely recapitulate key molecular features of human colorectal cancer, including alterations in Ki-ras, adenomatous polyposis coli (APC), transforming growth factor β, cyclin D1, and cyclin-dependent kinase 4 (11–20). The model provides an appropriate experimental system for studying molecular and pathological changes associated with the human disease. Importantly, inbred mice demonstrate differences in azoxymethane sensitivity. For example, A/J mice develop 10 to 20 tumors in distal colon, whereas tumors in AKR/J mice are rare (10, 11, 20). Interestingly, initiation of ACF upon carcinogen exposure is a feature common to both strains (5, 10, 18). However, a higher percentage of dysplastic ACF form in A/J colons compared with AKR/J. These observations provide the basis for additional stratification of ACF in terms of cancer risk, an approach that may eventually lead to identification of genes involved in early stages of colon tumorigenesis.

ACF are comprised of aggregates of abnormal crypts and characterized by hyperproliferation, increased size, expanded pericryptal zones, and elongated or slit-like crypt lumina (5, 10, 21, 22). A separate group received 0.9% NaCl (vehicle controls). Animals were sacrificed 6 weeks after the last injection and colons were flushed with PBS, embedded in cryoprotectant OCT, and frozen at −80°C. Eight serial cryosections (7-μm thick) with appropriate representations of preneoplastic lesions and abutting normal tissue were prepared. One section was stained with H&E for histologic classification of lesions, whereas the remaining six sections were stained using the HistoGene LCM staining kit (Arcturus, Mountain View, CA). Pure populations of epithelial cells comprising lesions were microdissected using the PixCell II LCM system (Arcturus). A summary of lesions is provided (Table 1). Because of the rarity of AKR/J dysplastic ACF, step-sections were prepared to obtain five lesions. Laser-captured saline-treated colonic epithelia were pooled and used as reference...
control, eliminating potential field effects that may exist within azoxymethane-exposed normal-appearing epithelium. The validity of this approach is demonstrated by similar expression profiles from laser-captured, saline-treated colonic epithelium from both strains (correlation coefficient, r = 0.86), validating their use as reference controls and avoiding potentially confounding effects of potentially heterogeneous expression profiles associated with independently captured adjacent normal epithelium from azoxymethane-treated mice. Approximately 50 to 1000 cells were laser-captured from each sample.

**ACF Classification.** Histologic evaluation was carried out on H&E-stained colon sections. The criteria used for histologic grading of ACF have been adapted from previous studies (10, 27-29) as follows: (1) hyperplastic: small (1 to 5 crypts/focus), moderately hypercellular, normal cellular differentiation with <30% loss of goblet cell differentiation, slight variation in crypt diameter relative to normal, basally arranged and slightly hyperchromatic nucleus; (2) dysplastic: moderate size (5 to 10 crypts/focus), markedly hypercellular, >50% loss of goblet cell differentiation; enlarged, often tortuous crypts with slit-shaped lumina, loss of polarity, dark, elongate to oval, hyperchromatic nuclei with pseudostatification and mitotic figures of 0 to 1 high power field; (3) microadenoma; large size (>10 crypts/focus) but not visible by the naked eye, severely hypercellular, complete loss of goblet cell differentiation, enlarged tortuous crypts; loss of cellular polarity, moderate nuclear anisokaryosis and mitotic figures of 1 to 2 high power field. ACF are defined as high- or marked dysplasia.

**RNA Extraction, Linear Amplification, and Array Hybridization.** Total RNA from 50 to 1000 laser-captured tumor cells and vehicle-treated control colon was extracted using the Picopure RNA isolation kit (Arcturus). Because ACF are generally comprised of 20 to 100 cells (400 to 800 pg of total RNA), colons was extracted using the Picopure RNA isolation kit (Arcturus). Because RNA from 50 to 1000 laser-captured tumor cells and vehicle-treated control and mitotic figures of 1 to 2/high power field. ACF are defined as high- or marked dysplasia; moderate size (5 to 10 crypts/focus), markedly nuclear anisokaryosis and mitotic figures of 1 to 2 high power field. ACF are defined as high- or marked dysplasia.

**Results**

**Histopathological Examination.** Colon sections were examined microscopically to confirm the histology of each lesion (Fig. 1). Significant numbers of ACF were observed in colons of both strains, although in A/J, a higher percentage of ACF was dysplastic (5, 10, 20). Hyperplastic ACF from both strains showed increased crypt size, moderate loss of goblet cell differentiation, low crypt multiplicity, and mild to moderate anisokaryosis (Fig. 1, A and C). Dysplastic ACF had increased crypt size, marked loss of differentiated goblet cells, increased crypt multiplicity, moderate to marked anisokaryosis, and nuclear pseudostratification. In addition, paneth cell metaplasia was noted in multifocal dysplastic A/J ACF (Fig. 1B).

**Immunohistochemical Profiling of ACF.** An initial attempt to discriminate populations of ACF from A/J and AKR/J colons was performed by IHC. Expression of several key tumor-related markers, including proliferating cell nuclear antigen, p53, β-catenin, APC, cyclin D1, and c-Myc, was evaluated (2, 11, 13, 20, 34-36). Deregulation of the Wnt signaling pathway, including mutations in the β-catenin gene and/or APC loss, has been implicated in colon tumorgenesis. Such alterations result in translocation of β-catenin from membrane to nucleus, with subsequent transcriptional activation of target gene targets, including cyclin D1 and c-myc. β-Catenin was detected at comparably high levels in the nucleus and cytoplasm of ACF from either strain, whereas adjacent normal-appearing and ve-
Vehicle-treated control colons showed only faint membrane staining (Fig. 1). However, there were differences in immunohistochemical scores for membrane staining of ACF. A/J ACF had a lower score relative to AKR/J (Fig. 1, G and H). Despite elevated nuclear staining of \( \beta \)-catenin, there were no mutations identified in either strain within the mutation prone glycogen synthase kinase-3\( \beta \) phosphorylation sites (data not shown). Because nuclear accumulation of \( \beta \)-catenin is often associated with loss of APC, we evaluated APC status using antibod-
ies specific to the NH2 terminus. As shown in Fig. 1, we found moderate elevation of APC, eliminating a causal relationship between APC loss and β-catenin accumulation. To rule out the possibility that accumulated APC protein has undergone truncation, tissues were probed with APC antibody specific for the COOH terminus. APC levels were increased in lesions, primarily localized to the nucleus, an observation that is consistent with the presence of full-length functional protein (37). Finally, no mutations were detected within the APC mutation cluster region in 10 A/J adenocarcinomas and ACF. These results suggest that β-catenin accumulation within ACF occurs independently of APC status. To assess the potential for differential activation of the Wnt pathway in A/J and AKR/J colons, we evaluated the status of c-myc and cyclin D1, two important β-catenin targets. Although elevated nuclear c-Myc and cyclin D1 were detected in ACF from both strains, staining intensity was comparable (Fig. 1).

While alterations in p53 nuclear staining are typically associated with later stages of colorectal cancer, the status of this tumor suppressor in ACF has not been extensively evaluated (11, 34, 35). Although p53 nuclear staining was consistently observed in ACF, the staining intensity was comparable between the two strains (Fig. 1, K and L). To extend our previous observations of accumulated wild-type p53 in A/J tumors (11), we carried out PCR-based sequence analyses (exons 5 to 8) of the p53 gene, using 10 laser-captured ACF from both strains and 5 A/J microadenomas. No mutations within this highly mutable region were identified. Thus, immunohistochemical analysis of key tumor biomarkers provided only minimal discrimination of high- and low-risk ACF.

LCM-Based Gene Expression Profiling. We next carried out a genome-wide, array-based gene expression analysis to identify unique and discriminatory molecular profiles. LCM was used to procure pure cell populations. Hyperplastic and dysplastic ACF, microadenomas, and adjacent normal epithelium were laser-captured (Fig. 2). Because ACF are comprised of only limited numbers of cells (20 to 500), the RNA yield is minimal (~50 to 500 fg/cell). Therefore, to circumvent the issues of contaminating cells and limiting RNA yield associated with these microscopic lesions, we combined the use of LCM with linear amplification (LCM-LA), using T7-based linear RNA amplification. This method routinely generated 30 to 70 μg of aRNA from laser-captured colonocytes (50 to 1000 cells). However, the size of ACF precluded verification of amplification linearity with a conventional comparison of nonamplified RNA versus amplified aRNA. Therefore, we analyzed expression profiles between nonamplified and amplified RNA from three adenomas and observed high correlation (r = 95). To additionally evaluate reproducibility of our labeling and hybridization strategies, eight random samples were collected in duplicate and hybridized to the array. The correlation coefficient of these duplicate experiments was r = 0.92. These results indicate that the T7-RNA amplification method preserves differential gene expression patterns. This methodology has been independently validated (31).

Gene Expression Profiling of ACF. Using LCM-LA, 50 colon specimens (Table 1; including random duplicates) resulted in 928,000 data points from 58 separate arrays. Single color hybridization with Cy5 enabled elimination of dye bias, a frequent problem in dual-color hybridization (31). This strategy, combined with the 75-percentile normalization method, enabled direct comparison between multiple arrays (32). The similarities in global expression profiles of salinetreated reference controls (r = 0.86) indicate that differences are an outcome of azoxymethane-induced alterations and not from inherent differences in basal gene expression between mouse lines (Fig. 3). To determine whether ACF could be segregated independent of histologic staging, we carried out cluster analysis on the entire data set (44 samples; refs. 30, 33). As shown in Fig. 4A, of 4896 genes interrogated by adaptive centroid algorithm, there was a clear separation between strains. Six general clusters (a-f, Fig. 4A) were identified
based on unique expression, allowing complete segregation of A/J and AKR/J lesions. The six gene clusters (Fig. 4A) contained a total of 226 genes in A/J ACF and 202 genes in AKR/J ACF that were significantly up- or down-regulated (2-fold) in at least one histologically defined subgroup. Although lesions did not show complete segregation, subsequent analysis of specific data subsets derived from histologically similar lesions showed clear separation. Although most of the microadenomas clustered together, it was difficult to segregate hyperplastic and dysplastic ACF; additional evidence that subtle differences in gene expression profiles are unique and therefore useful in discriminating low- and high-risk ACF. The expression level of each gene is represented by the ratio of gene intensity in the lesion relative to expression in the saline-treated control colons. The color-bar at the bottom correlates color intensity to gene expression.

Fig. 4. Cluster analysis of ACF from azoxymethane-treated mice using the adaptive centroid algorithm. A, dendrogram and heat map of the gene expression levels from hyperplastic ACF (H), dysplastic ACF (D), microadenoma (M), and adjacent normal-appearing (N) from azoxymethane-treated A/J and AKR/J colons, visualized using Genesite software (BioDiscovery). Of the 4896 genes represented on the array, 220 genes (rows) clustered into six broad clusters (A–F) showing similar and distinct expression patterns at different stages (columns) of colon tumor progression in A/J and AKR/J mice. Note that 126 of the genes identified were common to A/J and AKR/J ACF. B, Venn diagram of the total number of significantly altered (2-fold) genes that were identified by adaptive centroid algorithm from A/J and AKR/J colons. Although lesions did not show complete segregation into histologically similar lesions, additional evidence that subtle differences in gene expression profiles are unique and therefore useful in discriminating low- and high-risk ACF. The expression level of each gene is represented by the ratio of gene intensity in the lesion relative to expression in the saline-treated control colons. The color-bar at the bottom correlates color intensity to gene expression.
pression profiles characterize these lesions. There was obvious segregation of adjacent normal-appearing mucosa from ACF (Fig. 4A).

As shown in the Venn diagram (Fig. 4B), there was overlap of 126 genes between strains, demonstrating the likelihood of shared pathways in high- and low-risk lesions. Fig. 4C shows four representative gene subclusters with distinct expression profiles. These subclusters were selected on the basis of divergent expression patterns and include genes consistently up- or down-regulated across lesions of variable histology within mouse strains.

**Gene Profiles of Low- and High-Risk ACF.** To test the principal that ACF of similar morphology, but different risk potential, have unique molecular signatures, the following analyses were undertaken. Hyperplastic ACF were separated between high (A/J)- and low (AKR/J)-risk colons. Genes that are significantly and differentially regulated in Fig. 4 shows four representative gene subclusters with distinct expression profiles. These subclusters were selected on the basis of divergent expression patterns and include genes consistently up- or down-regulated across lesions of variable histology within mouse strains.

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**Table 2 Target genes validated by quantitative reverse transcription-PCR and their primer sequences**

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<tr>
<th>Account number</th>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Relative to control*</th>
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<td>A1413975</td>
<td>Rnh1</td>
<td>GTCAGGGCAGGAGGCAGCTGTGTT</td>
<td>GCCGCTAGGCTGGAGAGGAG</td>
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<td>A1427137</td>
<td>Psmd13</td>
<td>CAGTGTCGGCAGGAGGGAG</td>
<td>GCCACTTGTGAAGGCGAGAAG</td>
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<tr>
<td>A1323680</td>
<td>Iex-1</td>
<td>ATAGGTCGGTAAGCAGAGTGTGTT</td>
<td>CCGTGAAGCAGACACAGAACAG</td>
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<tr>
<td>A1324871</td>
<td>Thbs4</td>
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<tr>
<td>A1450297</td>
<td>EST</td>
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<td>TGCCCCCAAATTCATCCTTC</td>
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<tr>
<td>A1451408</td>
<td>Hes-V3</td>
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<tr>
<td>A1528651</td>
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<td>GCCTTGGTGAACACAGATGGGG</td>
<td>GCCTAGTACAGAAGCAGTCCA</td>
<td>2.07 ± 2.07 0.28 ± 0.33</td>
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* Expression levels of pooled saline control samples were used as calibrator. The values <1.00 indicate a decrease, and values >1.00 indicate an increase in gene expression relative to the control.

**DISCUSSION**

ACF can be stratified into two broad classes, dysplastic and hyperplastic, based in part on morphologic characteristics and predicted biological behavior (4, 5). Histomorphologic evaluation and grading of ACF represent one of the few available biomarkers of colon cancer risk (6–10). The present study was undertaken as an initial attempt to develop a molecular signature to discriminate ACF risk potential. IHC analysis of proliferating cell nuclear antigen, p53, and several components of the Wnt signaling pathway, including β-catenin, APC, cyclin D1, and c-Myc (2, 11, 13, 20, 34–36), afforded minimal discrimination of ACF. Surprisingly, intense β-catenin nuclear staining was observed in ACF from both strains. This accumulation occurred in the absence of mutations within the glycogen synthase kinase-3 phosphorylation site and was independent of APC loss. Increased nuclear staining was associated with elevated levels of several key β-catenin target genes, including cyclin D1 and c-myc, an effect that was not strain specific. The significance of the subtle difference in membrane staining of ACF between sensitive and resistant strains is presently under study.

Limited ACF discrimination by conventional IHC prompted a genome-wide, array-based expression analysis to identify discriminatory molecular profiles. Optimization of LCM-LA-MA afforded the opportunity to study gene expression patterns within individual colonic crypts. Although unsupervised clustering provided separation in the A/J and AKR/J colons, segregation of lesions based on histology was incomplete (Fig. 4A). However, within the complete data set, there were subsets of genes in both strains that were consistently altered by azoxymethane treatment. For example, in A/J colons, elevated levels of Pole2, Hes6, and sf3b1 were observed in hyperplastic and dysplastic ACF, microadenomas, and adjacent normal-appearing epithelium. Consistent alterations in these genes throughout the carcinogenic process imply a fundamental role in tumor progression. On the other hand, genes involved in immune response (Ubc-rs2, Psmd13, and Li), cell-cell adhesion (Mfge8), and angiogenesis (Rnh1) were elevated in ACF from the resistant AKR/J colons. Effective immune surveillance, contact inhibition, and controlled angiogenesis are likely to play a fundamental role in limiting ACF progression, and the additional interrogation of these pathways in human colorectal cancer may prove informative.

To test the principal that ACF of comparable morphology, but distinctly divergent risk potential, are characterized by unique molecular signatures, analysis of hyperplastic and dysplastic ACF was undertaken using adaptive centroid algorithm. These analyses enabled a correlation of transcriptional profile(s) within lesion subtypes with respect to morphologic stage, an approach that will enable our understanding of transcriptional programs that influence tumor progression or growth arrest of putative precancerous lesions. Because dysplastic ACF are associated with tumor progression, we focused our analysis on clusters representing genes that afforded maximal segregation across strains (Fig. 4E). Genes that are elevated in the A/J dysplastic ACF and down-regulated in the AKR/J dysplastic ACF include ASML3a, MAP-17, Rab24, SPI1, thrombospondin 4, and Grim-19.
as SLPI1 (secretory leukocyte protease inhibitor) and ASML3a (deleted in bladder cancer chromosome region 1) tumor suppressor gene (40). In fact, elevated expression of both genes was reported in human urinary bladder tumors (40), and it is possible that the high levels of ASML3a observed in dysplastic A/J ACF contribute to tumor progression. Several other discriminatory targets were also identified. MAP-17 was overexpressed in A/J dysplastic ACF, with only moderate levels present in morphologically matched AKR/J lesions. Although the precise role of MAP-17 has not been clarified, previous studies conducted in renal tubular epithelial cells have identified its association with the cytoplasmic aspect of the plasma membrane, implicating its role in cell-cell communication (41). In addition, abundant levels of MAP-17 have been detected within carcinomas arising from kidney, colon, lung, and breast (41). Although beyond the scope of the present investigation, additional studies to define the role of MAP-17 in human colon cancer is warranted. Rab24 (part of the Ras superfamily) is involved in vesicle transport, and we found that it was elevated in dysplastic A/J ACF. The potential significance of this observation with respect to colorectal cancer is based in part on earlier reports demonstrating high levels of this protein in hepatocellular carcinomas, prompting speculation that Rab24 may represent a proto-oncogene (42). Protease inhibitors have been associated with attenuation of tumor progression and metastasis. However, recent studies indicate that expression of secretory leukocyte serine protease inhibitors (SLPI1) may elicit an opposite effect, facilitating tumor progression, metastasis, and even a poor prognosis in humans (43). Interestingly, high levels of SLPI1 transcripts were observed in A/J dysplastic ACF, representing the first association of this serine protease inhibitor in colon tumorigenesis.

Cluster analysis identified a gene panel that was significantly induced in resistant AKR/J ACF. These genes include IEX-1, Mifge8, Rnh1, Psmul1, and Usf2 (Fig. 4E). The increased expression of a subset of genes involved in protein biosynthesis, DNA repair, transcription regulation, members of the nuclear factor-κB family, ion transport, and cell metabolism raise the possibility for establishing a gene signature for low-risk ACF. In fact, each of the genes represented within this cluster have putative functions that could potentially play a role in limiting ACF progression. For example, IEX-1 is induced by various cell stressors, including ionizing radiation, UV radiation, and growth factors (45). IEX-1, an nuclear factor-κB target gene, inhibits the activation of nuclear factor-κB, thereby countering its antiapoptotic potential (45). Whether IEX-1 elicits a comparable protective effect in low-risk ACF is certainly a possibility. To assess the potential relevance of IEX-1 in human colorectal cancer, we evaluated a total of 10 matched tumors with adjacent normal epithelium by IHC. As shown in Fig. 5, there was a marked reduction in IEX-1 staining within the tumor cells, suggesting the potential involvement of this immediate early gene in colon tumorigenesis. Additional analysis of this protein and its functional significance in human colorectal cancer is under way.

Although the importance of ACF as biomarkers of colon cancer risk is well established (5, 10, 36, 46, 47), few studies have attempted to additionally stratify these lesions at the molecular/genetic level. The incipient and dynamic nature of ACF is likely to complicate histology-based methods of prognostication. Additionally, it is known that early adenomas that are similar in appearance may be biologically distinct with varying rates of malignant conversion (46, 47). Thus, our primary objective was to identify unique transcriptional profiles of ACF at similar histologically defined stages before the onset of adenomas. Although one cannot rule out the possibility that the luminal environment may affect ACF outcome, it is clear from the identified gene clusters that ACF do, in fact, harbor distinct genetic profiles that may result in a divergence of phenotypes and subsequent tumorigenic potential. Interestingly, 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. Despite significant overlap, however, our data have identified a number of discriminatory targets that may affect the biological outcome of ACF. These data thus provide a proof-in-principle of our ability to stratify risk potential of ACF and provide the rationale for assessing the expression characteristics of a unique set of genes in human ACF.

Increased knowledge of gene expression patterns and risks inherent in ACF may additionally inform the relationship between human ACF
and tumorigenic potential. Patients with colorectal cancer demonstrate increased numbers of ACF (with greater percentages of large and dysplastic ACF) relative to patients with benign adenomas or those without evidence of pathology. These ACF are not uniformly distributed and cluster, both in cancer and polyp patients, within the distal bowel (48–50). Takayama et al. (51) have reported that in patients with adenomas and ACF, 96% of polyps were located in the left colon. The gene expression profiles from high- and low-risk mouse strains suggest that identification of molecular signatures from clinically accessible distal ACF may give new insight into cumulative risk for an individual or population at large. Thus, the use of predictive gene profiling as an adjunct to traditional histologic analysis permits the recognition of differences between histologies that may otherwise be difficult to stratify. By extrapolation, a similar approach to discriminate human ACF on the basis of expression profiles may provide enormous prognostic benefit in individuals at varying risk of colorectal cancer.

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