A Susceptibility Gene Set for Early Onset Colorectal Cancer That Integrates Diverse Signaling Pathways: Implication for Tumorigenesis

Yi Hong, Kok Sun Ho, Kong Weng Eu, and Peh Yean Cheah

Abstract

Purpose: The causative genes for autosomal dominantly inherited familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer have been well characterized. There is, however, another 10% to 15% of early onset colorectal cancers (CRC) in which the genetic components are unclear. In this study, we used microarray technology to systematically search for differentially expressed genes in early onset CRC.

Experimental Design: Young patients with non–FAP or non–hereditary nonpolyposis colorectal cancer, and healthy controls were age- (<50 years old), ethnicity- (Chinese), and tissue-matched. RNAs extracted from colonic mucosa specimens were analyzed using GeneChip U133-Plus 2.0 Array.

Results: Seven genes, CYR61, UCHL1, FOS, FOS B, EGR1, VIP, and KRT24, were consistently upregulated in the mucosa of all six patients compared with the mucosa from four healthy controls. The overexpression of these genes was independently validated with a testing set of six patients and six healthy controls. Principal component analysis clustered the healthy control specimens separately from the patient specimens. Real-time PCR quantification with SYBR-Green on nine other patient specimens not previously used in microarray assays confirmed the up-regulation of these seven genes. These genes function in a multitude of biological processes ranging from transcription, angiogenesis, adhesion, and inflammatory regulation to protein catabolism in various cellular compartments, from extracellular to the nucleus. They integrate known tumorigenesis (Wnt, PI3K, MAP kinase, hypoxia, G protein–coupled receptor), neurologic, insulin-signaling, and NFAT-immune pathways into an intricate biological network.

Conclusions: The data suggest that the patient’s mucosa is primed for tumorigenesis when cellular homeostasis is disrupted, and that the seven overexpressed genes could potentially predict early onset CRC.

Colorectal cancer (CRC) is one of the leading cancers in the developed world. In Singapore, it has the highest incidence among cancers and is the second leading cause of cancer death (1). Despite the recent advent in therapeutic intervention, the most promising strategy to reduce mortality and morbidity from the disease is still early detection (2). A study on twins has estimated that up to 35% of CRCs could be attributed to genetic factors (3). Germ line mutations in adenomatous polyposis coli and mismatch repair genes are responsible for the highly penetrant, and autosomal, dominantly inherited familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), respectively. Nevertheless, FAP and HNPCC comprise only 5% to 10% of total CRC incidence. There are at least another 10% to 15% of early onset CRCs for which the genetic susceptibility is unclear (4).

Accumulating evidence has alluded to the role of many low-penetrant modifying genes, such as the G870A single nucleotide polymorphism in cyclin D1, in increasing CRC risk (5, 6). However, no systematic study to ascertain the extent and cumulative risk purported to these susceptibility genes has been reported.

In recent years, microarray technology has increasingly been used to analyze various tumors including CRC (7–11). The microarray assay is a powerful tool for systematic and combinatorial search for molecular markers of cancer classification and outcome prediction (12). To our knowledge, however, no whole genome expression profiling of the germ line of patients with early onset compared with healthy controls has hitherto been done, probably because of the paucity of such well-defined sample sets.

In the present study, we did a genome-wide expression profile using complementary RNA from normal-appearing colonic mucosa of young patients without a family history of
the disease and compared that to the profile of age-, ethnicity-, and tissue-matched healthy controls. The aim was to ascertain a panel of differentially expressed genes that could potentially serve as a susceptibility gene set for early onset CRC.

**Patients and Methods**

**Patients and healthy control specimens.** Tumor specimens and adjacent grossly normal-appearing tissue at least 8 cm away were routinely collected and archived from patients undergoing colorectal resection at the Singapore General Hospital. Young (≤50 years old) Chinese patients whose tumors were classified as microsatellite-stable were included in this retrospective study. These patients did not have clinicopathologic features (retrieved from medical records) that fit the Bethesda criteria for HNPCC. Furthermore, the mucosa from these patients harbored three or fewer adenomatous polyps. Hence, these Chinese patients whose tumors were classified as microsatellite-stable were analyzed with the Affymetrix data mining tool. The cutoff criterion for gene calls was at least a 4-fold difference in expression between any two profiles compared, to avoid gene calls attributable to allelic imbalances or stochastic fluctuations. Differential expressions due to allelic differences were generally in the order of 1- to 4-fold (13). The microarray data set was submitted to the GEO repository (GSE4107) at http://www.ncbi.nlm.nih.gov/geo/info/linking.html.

**Quantitative real-time PCR analysis.** First-strand cDNA was synthesized from total RNA using the SuperScript First-Strand Synthesis System with SuperScript II reverse transcriptase according to the protocols of the manufacturer (Invitrogen, Carlsbad, CA). The cDNA generated was used as a template in real-time PCR reactions with QuantiTect SYBR-Green PCR master-mix (Qiagen, Hilden, Germany) and were run on an ABI PRISM 7700 machine. All PCR reactions were done in duplicate. Validation experiments verified that the efficiencies of amplification of the seven target genes and glyceraldehyde-3-phosphate dehydrogenase, the internal reference, were approximately equal. Hence, relative quantification or fold change between patients and the mean of four healthy controls for each gene (2^−ΔΔCt) were determined using the comparative Ct method. The assay was done on all 12 patient specimens previously arrayed and in 9 other patient specimens (five females and four males) similarly selected.

**Statistical methods.** Affymetrix data files were imported into “Spotfire” for further analysis. After filtering off genes (35%) that were not present in any of the specimens and outlying signal intensities with a magnitude >40,000 (1.1%), the remaining 35,120 probe sets were subjected to a 40-gene signature. The microarray data set was submitted to the GEO repository (GSE4107) at http://www.ncbi.nlm.nih.gov/geo/info/linking.html.

**Results**

**Expression profiling identifies differentially expressed probe sets and patient subgroups.** The complementary RNAs generated from both healthy controls and patient specimens were of high and comparable integrity. The mean percentage of transcripts scored as present and marginal were very similar between healthy controls and patients (Table 1). Furthermore, the mean 3'/5' ratios of the internal control and human glyceraldehyde-3-phosphate dehydrogenase for both the healthy controls and patient specimens were below the threshold of 3.0 (Table 1).

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**Table 1. Characteristics of patients and healthy controls microarrayed**

<table>
<thead>
<tr>
<th>Features</th>
<th>Patients (n = 12)</th>
<th>Healthy controls (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean (range)</td>
<td>40.9 (22-49)</td>
<td>39.5 (27-48)</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>7:5</td>
<td>5:5</td>
</tr>
<tr>
<td>Microarray report</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present gene call (mean %)</td>
<td>36.6</td>
<td>37.0</td>
</tr>
<tr>
<td>Marginal gene call (mean %)</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase 3'/5', mean (range)</td>
<td>(1.19-2.50)</td>
<td>(1.27-2.40)</td>
</tr>
</tbody>
</table>

Both patient and healthy control specimens were snap-frozen in liquid nitrogen within 30 min of removal from the colon and were stored at −80°C. This study was approved by the Ethics Committee of Singapore General Hospital.

**Microsatellite instability assay.** The HNPCC microsatellite instability test kit from Roche Molecular Biochemicals (Penzberg, Germany) was used to determine the microsatellite instability status of the patients' tumors. The panel included five microsatellite loci, BAT25, BAT26, D5S346, D17S250, and D2S123. Briefly, −200 ng of DNA from each matched mucosa and tumor tissues from each patient were used in each multiplex PCR reaction according to the manufacturer's protocol. A microsatellite-stable tumor is a tumor with identical profiles in all five loci compared with the matched mucosa tissue. A microsatellite-unstable tumor is a tumor with two or more loci with different profiles from mucosa.

**Genome-wide expression analysis.** Patients and healthy controls were divided into a training set and a testing set with approximately equal numbers. The training set was used to build a gene set which was subsequently validated using the testing set. Total RNA was extracted from each specimen and biotinylated complementary RNA targets were prepared with 5 μg of total RNA according to the manufacturer’s protocols (Affymetrix, Santa Clara, CA). Only RNA of high integrity (intensity of 285/18S RNA — 1.5) were used. No specimen pooling was done because the ultimate aim of the study was to identify biomarkers consistently differentially expressed across all individual patients compared with healthy controls. Targets were hybridized to GeneChip Human Genome U133 Plus 2.0 Arrays for 16 h. The hybridized arrays were washed and stained with streptavidin/phycocerythrin conjugate and anti-streptavidin antibody in Affymetrix Fluidic Station 450, and scanned with GeneChip scanner 3000. Signals were analyzed with the GeneChip operating system software. Chips were rejected if the marginal gene call was >2% and the 3'/5' ratio of the reference gene, human glyceraldehyde-3-phosphate dehydrogenase, was >3.0. Detection ("present", "absent", or "marginal"), change ("increased", "decreased", or "no change"), and fold change status (−log 2) between the signals generated from the mucosa of patients compared with the mucosa of healthy controls were analyzed with the Affymetrix data mining tool. The cutoff criterion for gene calls was at least a 4-fold difference in expression between any two profiles compared, to avoid gene calls attributable to allelic imbalances or stochastic fluctuations. Differential expressions due to allelic differences were generally in the order of 1- to 4-fold (13). The microarray data set was submitted to the GEO repository (GSE4107) at http://www.ncbi.nlm.nih.gov/geo/info/linking.html.
After filtering for detection (present) and change (increased) status, fold change analyses of patient specimens (px) compared with healthy controls (signalpx/signalmeanHC) were done using the data mining tool. Eight probe sets were consistently up-regulated at least 4-fold in all six patient specimens compared with the mean of the four healthy controls. In fact, for specimen 3416, the fold-change for one of the probe sets (representing FOS B) was >1,000-fold that of the four healthy controls (Table 3).

To validate this gene set, we arrayed a further testing set of six healthy controls and six patient specimens. The same eight probe sets were found to be consistently up-regulated in this testing set. All patient specimens exhibited at least 4-fold up-regulation in all eight probe sets compared with

![Image of hierarchical clustering and principal component analysis](image)

**Fig. 1.** Hierarchical clustering (A) and principal component analysis (B) for all specimens (n = 22). A, rows, differentially expressed genes; columns, specimens [left to right: columns 1-10, mucosa specimens from healthy controls (cluster 1); columns 11-22, mucosa specimens from patients (clusters 2 and 3)]. Red and green, relative high and low expression, respectively; *, specimen arrayed in the training set. B, Eigenvalues indicate that four principal components account for 95% of the variability; red circles, 10 healthy controls; blue circles, 12 patient specimens.

### Table 2. Seven consistently up-regulated genes in normal-appearing mucosa of human early onset CRC patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Genes</th>
<th>Biological process/molecular function</th>
<th>Cellular compartment</th>
<th>Implication in carcinogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>CYR61</td>
<td>Insulin-like growth factor–binding protein, regulation of cell growth, adhesion, angiogenesis, chemotaxis, wound healing, and morphogenesis</td>
<td>Extracellular</td>
<td>Overexpression of Cyr61 activates integrin-linked kinase-mediated Akt and β-catenin-TCF signaling pathway</td>
</tr>
<tr>
<td>(2)</td>
<td>UCHL1</td>
<td>Ubiquitin-dependent protein catabolism</td>
<td>Cytoplasm</td>
<td>UCHL1 is highly expressed in lung cancer. May contribute to p27 degradation</td>
</tr>
<tr>
<td>(3)</td>
<td>FOS</td>
<td>DNA binding, regulation of transcription from RNA Pol II; cell growth/maintenance, inflammatory response, development</td>
<td>Nucleus</td>
<td>FOS, FOS B, FOS L1, and FOS L2 are proto-oncogenes which are leucine zipper proteins that dimerize with proteins of the JUN family, forming transcription factor complex activating protein-1</td>
</tr>
<tr>
<td>(4)</td>
<td>FOS B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(5)</td>
<td>EGR1</td>
<td>Regulation of transcription—zinc ion binding</td>
<td>Nucleus</td>
<td>Neovascularization, microvascular endothelial cell growth, tumor angiogenesis, and tumor growth are processes critically dependent on Egr1</td>
</tr>
<tr>
<td>(6)</td>
<td>VIP</td>
<td>G protein – coupled receptor protein signaling, positive regulation of cell proliferation</td>
<td>Extracellular</td>
<td>VIP increases proliferation of cancer cells, elevates cyclic AMP and expression of c-fos, c-jun, c-myc, and vascular endothelial growth factor</td>
</tr>
<tr>
<td>(7)</td>
<td>KRT24</td>
<td>Structural molecular activity (intermediate filament). Influence cellular response to proapoptotic signals and the routing of membrane proteins in polarized epithelial cells</td>
<td>Membrane</td>
<td>Cytokeratin 20 and 7 protein and RNA expression are up-regulated in tumor cells. KRT is up-regulated during wound repair and in diseased skin. Mutations underlie several epithelial fragility disorders</td>
</tr>
</tbody>
</table>
the mean of the six healthy controls except for one probe set (representing KRT24) in specimens 3838 (0.2-fold) and 3894 (3.3-fold), respectively (Table 3). This anomaly could have been due to the loss of the particular transcript in these specimens.

There were 9,628 probe sets differentially expressed between patients and healthy controls according to t test analyses using the Spotfire software. Hierarchical clustering (using Euclidean distance) was then done (Fig. 1A). Specimens from patients and healthy controls were grouped into separate clusters. Interestingly, specimens from patients in cluster 3 (columns 16-22) whose tumors had clinical features associated with higher malignancies, or were later found to have metastasized, were grouped separately from the rest of the patients in cluster 2 (columns 11-15). For example, one of these patients in cluster 3 whose tumor was Dukes B (T3N0) at the time of the operation was subsequently found to have lung and liver metastases 9 and 18 months later, respectively. Thus, genome-wide expression profiling was able to differentiate the mucosa of patients from healthy controls as well as the mucosa of patients whose tumors were of higher malignancy from those of lower malignancy. All eight probe sets identified by fold change analyses were among the 9,628 probe sets identified by t test analyses.

We then did principal component analysis to verify whether these eight probe sets could group the specimens correctly. Normalized principal component scores clustered all 10 healthy controls together, whereas the patient specimens were more

Table 3. Fold change comparison in mucosa of early onset patients by microarray and real-time PCR assays

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>CYR61</th>
<th>EGR1</th>
<th>FOS</th>
<th>FOSB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Microarray</td>
<td>RT-PCR</td>
<td>Microarray</td>
</tr>
<tr>
<td>3335</td>
<td>111.6</td>
<td>44.0</td>
<td>30.9</td>
<td>14.9</td>
</tr>
<tr>
<td>3416</td>
<td>359.0</td>
<td>70.6</td>
<td>158.3</td>
<td>36.4</td>
</tr>
<tr>
<td>3446</td>
<td>171.6</td>
<td>37.3</td>
<td>37.3</td>
<td>11.6</td>
</tr>
<tr>
<td>3578</td>
<td>561.3</td>
<td>86.6</td>
<td>125.0</td>
<td>15.7</td>
</tr>
<tr>
<td>3798</td>
<td>107.8</td>
<td>131.4</td>
<td>32.9</td>
<td>30.9</td>
</tr>
<tr>
<td>3950</td>
<td>40.9</td>
<td>24.5</td>
<td>10.4</td>
<td>6.7</td>
</tr>
<tr>
<td>2965</td>
<td>379.7</td>
<td>87.9</td>
<td>24.4</td>
<td>21.1</td>
</tr>
<tr>
<td>3838</td>
<td>161.3</td>
<td>60.6</td>
<td>25.1</td>
<td>17.5</td>
</tr>
<tr>
<td>3216</td>
<td>78.5</td>
<td>30.9</td>
<td>15.2</td>
<td>11.7</td>
</tr>
<tr>
<td>3894</td>
<td>288.8</td>
<td>90.2</td>
<td>32.2</td>
<td>23.9</td>
</tr>
<tr>
<td>4145</td>
<td>66.2</td>
<td>30.3</td>
<td>32.9</td>
<td>8.5</td>
</tr>
<tr>
<td>4160</td>
<td>446.6</td>
<td>65.6</td>
<td>58.3</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Abbreviation: RT-PCR, real-time PCR assays.
*Indicates outliers.
heterogeneous but never overlapped with the healthy controls (Fig. 1B). K-means clustering (data not shown) also indicated that patient specimens clustered separately from the healthy controls. Therefore, the data suggests that these eight probe sets could differentiate the mucosa of patients from that of healthy controls. Eigenvector analysis indicates that the seven genes contributed equally to the principal components.

**Data mining the eight up-regulated probe sets.** These eight probe sets were fed into the NetAffy web site and Gene Ontology database for further annotation. They were found to comprise seven genes, i.e., cysteine-rich angiogenic inducer 61 (CYR61), ubiquitin COOH terminus esterase L1 (UCHL1), FBJ murine osteosarcoma viral oncogene homologue (FOS), murine osteosarcoma viral oncogene homologue B (FOS B), early growth response 1 (EGR1), vasoactive intestinal peptide (VIP), and keratin 24 (KRT24). CYR61 was represented by two probe sets. These seven genes were found to participate in various biological processes in multiple cellular compartments, ranging from the nucleus to the extracellular domain (14–24). Moreover, they were implicated in carcinogenesis to varying degrees (Table 2). When fed into the BiblioSphere PathwayEdition database, six genes were shown to link distinct signaling pathways into an intricate biological network (Fig. 2). The seventh gene, KRT24, is a novel gene that is separately linked to the Naegeli-Franceschetti-Jadassohn syndrome (24).

**Validation of the seven-gene set with quantitative real-time PCR.** To verify that the seven genes were indeed up-regulated in normal-appearing mucosa of patients compared with the healthy controls and were not an experimental artifact attributable to the microarray platform, a second independent assay was conducted. Quantitative real-time PCR done on all 12 patient specimens previously arrayed, and 9 other patient specimens, indicated that the seven genes were consistently up-regulated in all 21 patient specimens. In fact, real-time PCR seemed to be a more sensitive assay than microarray assays, as the fold change analysis for the former was generally higher than the latter (Table 3), which is in agreement with the findings of earlier studies (25). Regression plot analyses for the 12 patient specimens also shows that, except for a few outliers, the fold change by both microarray and real-time PCR assays for the seven genes independently (Fig. 3A-G) or combined (Fig. 3H) were positively correlated.

## Discussion

Hitherto, it was generally assumed that normal-appearing mucosa adjacent to a tumor in the patient’s colon was metabolically normal. Previous studies have often used such “matched” tissue as a baseline for comparisons with tumor. We show in the present study that the gene expression levels in morphologically normal-appearing mucosa from patients with cancer were vastly different from that of healthy controls, suggesting that the mucosa of patients were actually not normal but were already “primed” for carcinogenesis (Fig. 1A).

Stringent criteria were adopted to select for potential markers of early onset CRC without dominant family history. Patients and healthy controls were homogeneous with respect to age and ethnicity, and were tissue-matched to minimize artifacts attributable to age, ethnicity, or tissue-specific expression. Furthermore, only non–FAP and non-HNPCC patient specimens were included to minimize sample heterogeneity. To minimize variability due to stochastic fluctuation (26), the signal from each patient was compared with the mean of several healthy controls. The order in which the healthy controls and patients specimens were arrayed was also randomized to avoid bias from signals that could inadvertently be introduced by the machine (27).

Seven consistently up-regulated genes selected from a training set were validated by a completely independent testing set. The microarray data were further validated by quantitative real-time PCR assay (Table 3; Fig. 3), as suggested by the conclusions of earlier studies (9, 10).

These seven genes play key roles in diverse biological processes (Table 2) such as transcription regulation (FOS,
Fig. 3. Regression plots for fold change by microarray (Y-axis) and quantitative real-time PCR (X-axis) assays for the seven genes individually (A-G) or combined (H).

A, outliers (A-E-G); outliers are points >2 SD away from the regression line; ■ and ◇, KRT24 and all other genes, respectively (H).
FOS B, EGR1), angiogenesis (EGR1, VIP, CYR61), apoptosis (KRT-24), adhesive, migratory, and inflammatory signaling (CYR61, KRT24), and protein catabolism (UCHL1). Furthermore, these genes integrate not only known colorectal tumorigenesis pathways such as the Wnt, PI3K, MAP kinase, hypoxia, and G protein–coupled receptor signaling pathways, but also the insulin-signaling (glucose metabolism), the Ca2+/NFAT immune, and the human gastrointestinal nervous system via the UCHL1 (PGP9.5)-VIP-EGR1 link (Fig. 2). The latter pathways have generally not been previously regarded as important for the transformation to the cancerous state. Thus, the data suggests that a general disruption of signaling and metabolic pathways is essential for tumorigenesis to occur.

This is consistent with accumulating evidence that early onset CRC among young patients without a dominant family history of the disease was a result of the cumulative effects of many susceptibility genes (4, 28) rather than highly penetrant mutations in a tumor suppressor or oncogene. Although these seven genes may not necessarily be the same genes in which combined effects initiate the tumorigenic process, this panel of genes was the most sensitive to perturbation, and hence, could potentially serve as biomarkers for early changes in the mucosa of individuals at risk. In this regard, it is interesting to note that although FOS and EGR1 (Fig. 2) seem to be the signal transducers or transcription factors that link the various pathways into a network, the other five genes contribute equally to the principal components of the analyses. The data thus imply that all seven genes could potentially be targets for therapeautic intervention.

The only specimen from a patient with a right-sided tumor (Fig. 1B) was within the patients’cluster in the principal component analysis, suggesting that the seven genes were able to differentiate the mucosa of a patient from healthy controls irrespective of the eventual site of tumor development.

To our knowledge, there is only one previous study reporting differential expression by quantitative real-time PCR from 5 out of 15 genes examined in the mucosa of patients compared with healthy controls (29). The patients and controls were not age- and ethnicity-matched, and the 15 genes were not preselected by whole genome profiling but were culled from previous reports. We examined the expression of these five genes in our series. Only cyclooxygenase-2 and proliferating peroxisome-activating receptor-γ were up-regulated and down-regulated, respectively, in all patient specimens. These two genes were not included in our list because they were <4-fold differentially expressed in some of the patient specimens compared with healthy controls.

In summary, we have shown in this study that the expression of a large proportion of genes from the mucosa of patients differed from that of the healthy controls. Use of patients’ matched mucosa as a baseline in gene expression studies for carcinoma is likely to underestimate the degree of altered regulation. We further show that seven genes were consistently up-regulated in all patients’ mucosa compared with healthy controls using two independent assays, microarray assays and quantitative real-time PCR. These genes are key players in multiple biological processes in several cellular compartments and link diverse pathways into a biological network. The overexpression of these genes could serve as the signal for cell-wide disintegration of homeostasis and “priming” of the colonic epithelium for the cancerous state. Therefore, the findings imply that these seven genes could potentially serve as a susceptibility gene set for early onset CRC.

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References


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