Transcriptional Dynamics in Colorectal Carcinogenesis: New Insights into the Role of c-Myc and miR17 in Benign to Cancer Transformation

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Abstract

Colorectal cancer develops in a sequential, evolutionary process, leading to a heterogenic tumor. Comprehensive molecular studies of colorectal cancer have been previously performed; still, the process of carcinogenesis is not fully understood. We utilized gene expression patterns from 94 samples including normal, adenoma, and adenocarcinoma colon biopsies and performed a coexpression network analysis to determine gene expression trajectories of 8,000 genes across carcinogenesis. We found that the majority of gene expression changes occur in the transition from normal tissue to adenoma. The upregulated genes, known to be involved in cellular proliferation, included c-Myc along with its targets. In a cellular model system, we show that physiologic upregulation of c-Myc can lead to cellular proliferation without DNA replication stress. Our analysis also found that carcinogenesis involves a progressive downregulation of genes that are markers of colonic tissue and propose that this reflects a perturbed differentiation of colon cells during carcinogenesis. The analysis of miRNAs targets pointed toward the involvement of miR17 in the regulation of colon cell differentiation. Finally, we found that copy-number variations (CNV) enriched in colon adenocarcinoma tend to occur in genes whose expression changes already in adenoma, with deletions occurring in genes downregulated and duplications in genes upregulated in adenomas. We suggest that the CNVs are selected to reinforce changes in gene expression, rather than initiate them. Together, these findings shed new light into the molecular processes that underlie the transformation of colon tissue from normal to cancer and add a temporal context that has been hitherto lacking.

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Introduction

Colorectal cancer is the third most common cancer, and it is one of the main causes of cancer mortality worldwide (1). Colorectal cancer commonly starts as adenoma polyps, which are developed to adenocarcinoma, and in a later stage to invasive cancer. The development of colorectal cancer takes several years: 2 to 5 years for the maturation of advanced adenoma and additional 2 to 5 years to develop into early cancer, which can be morphologically and histologically characterized and classified. Fortunately, removing of adenoma polyps in the early stages decreases the risk of colorectal cancer (2).

In addition to the morphologic changes, the colorectal cancer development is characterized by specific molecular signature. The adenomatous polyposis coli (APC) gene was found to be mutated in approximately 70% to 80% of sporadic colorectal adenomas and carcinomas (3). Similarly, mutations in KRAS and TP53 genes are found in 40% and 60% of tumors, respectively. Furthermore, recent studies revealed that 32 genes are recurrently mutated in colorectal cancer (3). Many of the mutations accumulate in a sequential manner. For example, APC plays a pivotal role in early adenomas, mutations in KRAS are found in advanced adenomas and TP53 mutations are important for the carcinoma transduction (4).

The genes mutated in colorectal cancer are known to be involved in many different cellular processes. Therefore, to shed further light into the relationship between the genetic alterations and carcinogenesis, large integrative genome-wide studies can be used to move from the level of a single gene to a system or a pathway. Such a large-scale study recently explored the molecular signature of colorectal adenocarcinomas by profiling gene and miRNA expression, methylation levels, copy-number variations (CNV), and other genetic mutations by DNA sequencing (3). Overall, five pathways were found to be frequently altered in the adenocarcinoma, including WNT,
TGFB, RTK/RAS, PI3K, and TP53 pathways (3). Furthermore, integrative pathway analysis pointed to c-Myc (MYC) as a pivotal pathway in colon adenocarcinoma (3). However, this study looked at the end point of carcinogenesis, the colon adenocarcinoma itself, and the question remains at what stage in carcinogenesis these pathways exert their effect. Only few studies have looked at the temporal dynamics of colon cancer progression, but even those studies were limited to the normal to adenoma transition or the adenoma to carcinoma transition (5–8).

In this study, we set to answer the following questions: First, can we differentiate processes that occur early in cancer progression, already at the adenoma stage, from processes that are cancer specific? Second, can we identify potential drivers that underlie these processes? Finally, what is the relationship between genomic alterations found in colorectal cancer and change in gene expression? To answer those questions we used gene expression profiling from 94 different colon samples, normal, adenoma, and adenocarcinoma, to survey global changes in gene expression during colon cancer progression. By performing a network analysis, and juxtaposing the results against recent high-throughput analyses, we were able to place on a chronologic axis events that occur in colon carcinogenesis.

Materials and Methods

Acquisition and processing of expression data

All data analysis was performed using the R Project for Statistical Computing (http://www.r-project.com). All plotting was done using the R package ggplot2 (9) unless otherwise specified. Raw expression data was acquired from the Gene Expression Omnibus (accession GSE37364; ref. 8). CEL files were processed using the rma function from the affy package (10) using curated annotations available from the Brainarray resource (11). Probes not corresponding to genes were removed. We limited our analysis only to genes that were expressed (RMA value ≥5) in all samples from at least one of the stages (normal, early adenoma, late adenoma, early colorectal cancer, and late colorectal cancer), leaving 14,647 genes. Hierarchical clustering on the samples was performed and visualized using the heatmap.2 R function available in the gplots package.

WGCNA network

We used the WGCNA R package (12) to perform a signed weighted gene coexpression network analysis (13). We took 8,000 genes for the network analysis with the highest interquartile range (IQR). To construct the network, first, an adjacency matrix was calculated, by raising the correlation matrix to a power determined using scale-free topology criterion. A topology overlap matrix (TOM) was calculated on which hierarchical clustering was used to determine the modules. Modules that were correlated (r > 0.75) were merged. As one gene (LEN68) did not fit any module, it was removed, leaving 7,999 genes distributed across 9 modules. To represent modules using a single gene, the first eigenvector of the correlation matrix of the genes in the module (the module's "Eigengene") was used. To determine the membership of each gene in the module, the average connectivity (the average TOM value) of the gene in the module was used.

Functional annotation of the modules

Enrichment in the modules for functional annotations was determined using the ToppCluster web server (14). The server uses the ToppGene tool to perform the enrichment analyses, and the databases used for the Gene Ontology (GO), pathway, miRNA, and transcription factor-binding sites are described in the ToppGene publication (15). Wordcloud was generated using the wordcloud R package.

Cell Cultures and c-Myc expression

Telomerase-negative normal human diploid foreskin fibroblast cells (Bj cells) expressing a transfectected hTERT (TERT; ref. 16) were grown in DMEM supplemented with 10% FBS, 100,000 U/L penicillin, and 100 µg/L streptomycin. For c-Myc expression, either the pWZL Blast myc (Addgene plasmid 10674; ref. 17) or GFP retroviral plasmid were utilized. The plasmid was transiently transfected to Phoenix retroviral packaging cells (18). Bj-hTERT passage 53 cells were infected three times with the Phoenix cell supernatant, which contained the replication-defective virus. Then, the infected cells were selected using 6 µg/mL of Blastidicin S for the next 10 days.

Molecular combing

Unsynchronized BJ-hTERT were pulse labeled for 30 minutes by a medium containing 100 µmol/L of the thymidine, analog iododeoxyuridine (IdUrd). At the end of the first labeling period, the cells were washed twice with a warm medium and pulse labeled once more for 30 minutes with a medium containing 100 µmol/L of another thymidine analog, chlorodeoxyuridine (CldU). Cells were then harvested and genomic DNA was extracted, combed, and analyzed as previously described (19, 20). The primary antibody for fluorescence detection of IdUrd was mouse anti-Brdurd (Becton Dickinson), and the secondary antibody was goat anti-mouse Alexa Fluor 488 (Invitrogen). The primary antibody for fluorescence detection of CldU was rat anti-CldU (Novus Biologicals). The secondary antibody was goat anti-rat Alexa Fluor 594 (Invitrogen). The anti-Brdurd was used in a 1:5 dilution; all other antibodies were used in a dilution of 1:25 in blocking solution (Invitrogen). The length of the replication signals was measured in micrometers and converted to kilo bases according to a constant and sequence-independent stretching factor (1 µm = 2 Kb), as previously reported (21).

Determining enrichment for gene sets

Enrichment was determined using the Fisher exact test. ORs, P values, and 95% confidence intervals were calculated using the fisher.test R function.

Correlating miRNA expression with targets

Gene and miRNA expression were acquired from the TCGA Data Portal (https://tcga-data.nci.nih.gov/tcga/). For each of the miRNAs with enrichment of targets in the Early 2 module, the list of target genes in the modules was determined. Then, the correlation between each of the miRNAs and these targets...
was calculated, with \( P \) value determined using the T distribution and corrected for multiple testing using false discovery rate (FDR).

**Distribution of CNVs between the modules**

Regions enriched with CNVs in colorectal cancer was acquired from a published study (3). Only CNVs that were significantly enriched (FDR corrected \( q \) value < 0.05) were taken for enrichment analysis. Enrichment in the different modules was determined using Fisher exact test, as implemented in the R fisher.test function.

**Results**

The gene expression program changes more profoundly in the transformation from normal tissue to adenoma

To study normal–adenoma–carcinoma transformation, we utilized patterns of colon biopsy gene expression derived from 94 individuals (38 healthy controls, 16 low-grade adenoma, 13 high-grade adenoma, 14 Dukes’ A–B adenocarcinoma, 13 Dukes’ C–D adenocarcinoma; ref. 8). After normalization and removal of nonexpressed genes, we determined how the global dynamics of gene expression correlate with the different stages in carcinogenesis. To this end, hierarchical clustering on the 1,000 most variable genes (determined as the genes with the highest IQR) was performed and visualized as a heatmap (Fig. 1). We found that gene expression divided the samples mainly into two groups that showed a profoundly different expression pattern: normal colon tissue and nonnormal tissue, including both adenoma and adenocarcinoma. While gene expression by and large also separated adenoma from adenocarcinoma, the differences were much less pronounced, and the different tumor subtypes were not well defined. This suggests that a major shift in the transcriptional program occurs in the transition from normal tissue to adenoma followed by smaller but important changes at later stages.

**Activation of cell proliferation occurs early in colon carcinogenesis and is likely regulated by c-Myc**

To further explore the dynamics of gene expression across colon cancer development, we performed a signed weighted gene coexpression network analysis (WGCNA; refs. 12, 13). This analysis identified nine network modules, ranging from 104 to 3,516 genes (Supplementary Table S1). We plotted the expression of a simulated representative gene (hereafter “eigengene”) from each module across the different stages (Supplementary Fig. S1). The modules represented four different expression trajectories: (i) genes upregulated in the adenoma (module Early 1), (ii) genes downregulated in the adenoma (modules Early 2–3), (iii) genes upregulated in carcinoma (modules Early 1–3), and (iv) genes downregulated in adenoma with recovery of expression in carcinoma (modules Late 4–5). Similar to the analysis above showing that most of the changes in gene expression occur in the transformation from normal tissue to adenoma, the two early response modules were the largest. The first module (Early 1) had 3,516 genes and the second (Early 2) had 2,684 genes, together accounted for 77% of the genes in the network. To functionally annotate the modules, we used the ToppCluster webserver (14), restricting the results to GO terms, pathways, interactions, miRNA, and transcription factor targets (Supplementary Table S2).

We first focused on the largest module, Early 1, showing a sharp increase between the normal tissue and adenoma, followed by stable expression pattern throughout the different stages in carcinogenesis (Fig. 2A). Functional annotation of this module showed that it was enriched with terms related to cell proliferation, including cell cycle, DNA replication, nucleotide metabolism, check point, and DNA repair pathways (Fig. 2B; Supplementary Table S2). Transcription factor analysis revealed enrichment of genes regulated by the E2F transcription factors that regulate transcription of genes involved in the cell cycle (Supplementary Table S2). Significant enrichment was also found for genes upregulated by activation of c-Myc (Supplementary Table S2). This included genes harboring the c-Myc CACGTG E-Box as well as genes harboring the NNACGTGGTNN c-Myc–associated motif. Enrichment was also found for validated targets of c-Myc transcriptional activation, a pathway curated by the National Cancer Institute. As c-Myc regulates the expression of 10% to 15% of genes in the human genome (22), we wanted to study whether the induction of c-Myc in the adenoma was concurrent with genes found to be upregulated by c-Myc specifically in cancer tissues. To do so, we compared the distribution of genes in the network with genes upregulated in primary breast cancer cells in culture overexpressing c-Myc (23). We found a very significant enrichment of cancer-specific c-Myc targets in the Early 1 module (Fig. 2C). Of 108 genes upregulated in c-Myc–overexpressing cells (which were also included in our network), 84 (77%) genes belong to the Early 1 module, representing more than a 4-fold enrichment (OR = 4.46; \( P = 6.5 \times 10^{-13} \) in two-sided Fisher exact test). Together, these results indicated that c-Myc specifically drives cell proliferation already in early adenomas and along carcinogenesis.

**Mild upregulation of c-Myc induces coordinated DNA replication**

Our analysis of the Early 1 module suggests that upregulation of c-Myc coordinately activates many replication-associated pathways. On the other hand, in vitro studies showed that high c-Myc expression leads to perturbed proliferation and replication stress (24, 25). However, a more physiologic, mild overexpression (3.2-fold) of c-Myc by a retroviral vector in immortalized fibroblast cells (BJ-hTERT) was previously shown to increase cellular proliferation (26). To test whether mild overexpression of c-Myc also leads to replication stress, we studied the replication dynamics using DNA combing (Fig. 3). We found that moderate c-Myc overexpression did not affect fork rate progression (c-Myc, 1.02 ± 0.032 Kb/min, \( n = 175 \); Vector, 1.00 ± 0.04 Kb/min, \( n = 94 \)), and fork symmetry (average ratio between forks: c-Myc, 0.75 ± 0.02, \( n = 53 \); vector, 0.77 ± 0.03, \( n = 34 \); Fig 3B and C). Previously, overexpression of c-Myc was shown to lead to increased origin activation in different in vitro models, leading to genomic instability (24, 25).

We analyzed origin density using the well-established measurement of outgoing sister forks (27, 28). Our analysis revealed nonsignificant differences between c-Myc–upregulated cells
and control cells (c-Myc, 96.1 ± 5.3 Kb, n = 65; vector, 85 ± 7.4 Kb; Fig. 3D). These results indicate that moderate c-Myc upregulation, similar to the levels observed in the adenoma and adenocarcinoma samples, has the ability to lead to coordinated DNA replication without replication stress.

Perturbed cellular differentiation starts early in carcinogenesis and is accelerated during the adenoma–carcinoma transition

The other large module, changing already at the adenoma stage (Early 2), showed a consistent decrease throughout carcinogenesis (Fig. 4A). Functional annotation of the module found enrichment with genes involved in fatty acid metabolism and other metabolic processes (Supplementary Table S2). This result seemed puzzling at first, as upregulation of fatty acid metabolism is essential for cell proliferation and cancer progression (29). Cell-type signature analysis, using a publicly available database of cell type–specific markers (30), revealed that Early 2 module was most enriched with genes highly expressed in colonic tissue ($P = 5.9 \times 10^{-24}$). More than 60% of the markers for colon belong to the Early 2 module, which is the highest enrichment and most significant among the modules (Fig. 4B). Furthermore, we found that the enrichment for fatty acid catabolism and colon markers originated from the same genes, as the genes associated with carboxylic acid catabolism were themselves enriched for colon markers ($P = 7.8 \times 10^{-11}$).

To identify potential drivers, we conducted a miRNA target enrichment analysis. We found that the Early 2 module was enriched with gene targets of many known miRNAs (Supplementary Table S2), including miRNAs that are known to be associated with cancer (oncomiRs). Among them are the miR17 (MIR17) and miR27 (MIR27A and MIR27B) families, which regulate cell proliferation and differentiation. As this analysis is largely based on predicted miRNA gene targets, we asked whether we could provide additional support for the link between these miRNAs and gene expression in the context of colon cancer. As our dataset did not include miRNA measurements, we used published RNA-seq and miRNA-seq from colon adenocarcinoma samples (3). For each of the miRNAs, we calculated the correlation between the expression of the
miRNA and its putative targets within the Early 2 module (Fig. 4C). The most significant correlation was for the oncomiRs, miR17, showing a strong negative correlation with its putative targets ($r = -0.4, P = 4.2 \times 10^{-4}$). This result suggests that miR17 may contribute to the downregulation throughout carcinogenesis of genes in the Early 2 module.

**Activation of genes involved in angiogenesis, cell mortality, and immune response is cancer specific**

In addition to the Early modules, five other modules showed specific activation in the adenocarcinoma stages (Modules Late 1–5 in Supplementary Fig. S1). Of these, two modules, the Late 3 module (154 genes) and the Late 4 module (127 genes), were highly enriched with genes involved in inflammatory, and innate immune response pathways, including the Toll-like receptor signaling pathway, positive regulation of NF-kB, and MAPK activity. Furthermore, functional analysis of the Late 3 module also revealed enrichment to genes involved in regulation of apoptosis in the tumor. Interestingly, the Late 3 module represented genes with constant expression in the normal and precancer stages, and specific upregulation in cancer. On the other hand, the Late 4 module represented genes downregulated in the adenoma followed by upregulation in the carcinoma. The other three cancer-specific modules Late 2 (446...
genes), Late 1 (246 genes), and Late 5 (104 genes) were enriched with genes involved in angiogenesis and changes in the extracellular matrix. In addition, module Late 2 was enriched for gene targets of miR29c (MIR29C), which was recently found to be downregulated in gastric cancer (31). Most of the targets of miR29c in the module were genes that encode collagen proteins, which play an important role in the organization and function of the extracellular matrix in normal as well as cancer cells (32). Taken together, these results show that changes in gene expression reflect changes in the microenvironment of the tumors.

Genomic alterations reported in adenocarcinoma occur in genes that change their expression early in carcinogenesis

Genomic alterations associated with genomic instability are a hallmark of carcinogenesis (33). Genomic alterations, including whole chromosome and whole arm loss, as well as smaller CNVs, were reported to occur as a late event in colon carcinogenesis (34). We sought to determine whether the expression of genes that reside in these alterations was similarly changing only at later stages in carcinogenesis. As whole-arm chromosomal aberrations tend to include many different genes, we concentrated on the smaller CNVs found to be significantly enriched across different adenocarcinoma samples (3). We also separated the CNVs to duplications and deletions, as the expression effect should be opposite (Fig. 5). Surprisingly, despite the fact that the CNV events are known to occur at a later stage, genes in both duplications and deletions tend to be in modules that show an early change in carcinogenesis. Furthermore, the direction of the changes in expression was consistent with the type of CNV (duplication or deletion). Genes in duplications tend to be upregulated in the adenoma, consequently significantly enriched in Early 1.
module (OR, 1.79; \( P = 0.016 \) in two-sided Fisher exact test) and depleted in the Early 2 module (OR, 0.47; \( P = 0.010 \) in two-sided Fisher exact test), while genes within deletions show the opposite trend: downregulated in the adenoma, and enriched in the Early 2 module (OR, 1.49; \( P = 0.0010 \) in two-sided Fisher exact test).

**Discussion**

Several notable findings emerge from our analysis of gene expression trajectories in colon carcinogenesis. First, we found that the majority of gene expression changes occur between normal and adenoma samples. Our network analysis attributed 82.9% of the genes to one of the Early trajectories, and only 13.4% were attributed to a Late trajectory. This result indicates that the adenoma–carcinoma transition incorporated a relatively small amount of specific changes, a finding that is in agreement with previous results (5, 6).

Second, we found evidence for activation of coordinated cellular proliferation driven by c-Myc. This activation was characterized by activation of the pre-replication and DNA replication machinery. This result suggests a different oncogenesis mechanism in colorectal cancer relative to cervical cancer induced by human papillomavirus, or Cyclin E oncogene. In cervical cancer, the viral oncogenes E6 and E7 drive cellular proliferation by activation of the E2Fs, however, c-Myc remains unaltered, leading to uncoordinated cellular proliferation and DNA replication (26). This result appears to contradict reports that found that *in vitro* c-Myc expression perturbs replication (24, 25). However, in these studies, c-Myc was very dramatically overexpressed, whereas our analysis revealed that in early adenoma c-Myc expression increase to about 4-fold, and remained at this level along carcinogenesis. Accordingly, we found that inducing approximately 3-fold increase in c-Myc expression in fibroblasts leads to increased cellular proliferation without replication stress (Fig. 3). Therefore, we suggest that replication stress in colorectal cancer is not an early event but rather results from changes that occur later in carcinogenesis. This observation is supported by the recent finding that the loss of chromosome 18q, an event known to characterize adenocarcinomas and not adenomas, is involved in replication stress in colorectal cancer (35). Together, our results indicate that cell proliferation driven by c-Myc is characteristic of early colorectal cancer development, but its contribution to the transition from adenoma to adenocarcinoma is limited. In addition to cellular replication, we found concurrent upregulation of genes involved in DNA damage and cell-cycle checkpoints. As many of the genes involved in DNA damage response are activated by posttranslational modification (36), we believe that their increased expression does not indicate DNA damage and genome instability at this early stage.

Third, we found a progressive downregulation of colon-specific genes throughout carcinogenesis. Cellular proliferation and differentiation are in many cases opposite processes (37). The state of tumor differentiation is a well-known method of classification of tumor grade; however, molecular markers for grade determination are not yet available. Previous evidence linked the miR17 family to different types of cancer, including colorectal cancer (38, 39). Our results suggest that miR17 family play a pivotal role in the regulation of colon-specific genes. Importantly, the miR17 family is known to be positively regulated by c-Myc (40). This provides a link between the upregulated and downregulated Early modules, emphasizing the role of c-Myc as a driver of early carcinogenesis. Other studies found that the miR17 family promotes cancer development by regulation of cell proliferation, apoptosis, and angiogenesis (38). On the basis of our results, we suggest new roles for miR17 in carcinogenesis, either in perturbing the differentiation of colon cells or by contributing to dedifferentiation of cells in the tumor at early stages of cancer development.

A fourth notable as well as surprising finding was that genes located in CNV regions described in advanced colorectal cancer (3) change their expression already at the adenoma stage. This change was in the same direction as the later occurring CNV, with downregulated genes occurring in deleted regions and upregulated in duplicated regions. MYC itself is an example of such gene that is upregulated in the adenoma, and later in the colorectal cancer was found to be amplified. Genomic instability is a hallmark of cancer (41, 42), with losses and gains of chromosomal region affecting gene expression, and playing an important role in carcinogenesis, including colorectal cancer (43). Copy-number changes are a characteristic of advanced adenomas (4, 44). Our results suggest a mechanism in which the late occurring CNVs act to cement and reinforce changes in gene expression that occur at the early stages of tumorigenesis.

Our study has some obvious limitations. Above all, while our analysis could identify pathways changing in the transitions across carcinogenesis, functional annotation alone can only suggest the gene drivers underlying the observed changes, not tie them conclusively. For these reasons, functional experiments, such as the one performed in this study for c-Myc, are crucial, as they can analyze, in a controlled study, processes observed in the tumors. Similar to this, studies of other potential drivers identified in this study should be further studied. Another limitation is that our samples in different stages of carcinogenesis are from different individuals, making our analysis potentially vulnerable to inter-individual expression differences. These differences are evidenced by the considerable heterogeneity in the expression of the modules between samples of the same stage (Supplementary Fig. S1). Indeed, the modules cannot be used on their own for classification of tumor biopsies. However, we think that our focusing on the largest overarching trends of gene expression changes during carcinogenesis goes a long way to mitigate this concern.

Despite these limitations, our study provides important novel insights into the process of colon carcinogenesis and raises attractive hypotheses for further study. Specifically, the mechanisms underlying the activation of cellular proliferation by c-Myc, the proposed involvement of miR17 in cell dedifferentiation, and the mechanism by which differentially expressed genes acquire copy-number alterations are important questions arising from this research. Answering those questions will provide further insights into the processes of carcinogenesis.
and have potential implications to cancer prevention and therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: E. Ben-David, A. Bester, S. Shifman, B. Kerem
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