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Bioinformatics analysis of RNA-seq data revealed critical genes in colon adenocarcinoma

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Abstract. – OBJECTIVE: RNA-seq data of colon adenocarcinoma (COAD) were analyzed with bioinformatics tools to discover critical genes in the disease. Relevant small molecule drugs, transcription factors (TFs) and microRNAs (miRNAs) were also investigated.

MATERIALS AND METHODS: RNA-seq data of COAD were downloaded from The Cancer Genome Atlas (TCGA). Differential analysis was performed with package *edgeR*. False positive discovery (FDR) < 0.05 and |log2 (fold change)|>1 were set as the cut-offs to screen out differentially expressed genes (DEGs). Gene coexpression network was constructed with package *Ebcoexpress*. GO enrichment analysis was performed for the DEGs in the gene coexpression network with DAVID. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed for the genes with KOBASS 2.0. Modules were identified with MCODE of Cytoscape. Relevant small molecules drugs were predicted by Connectivity map. Relevant miRNAs and TFs were searched by WebGestalt.

RESULTS: A total of 457 DEGs, including 255 up-regulated and 202 down-regulated genes, were identified from 437 COAD and 39 control samples. A gene coexpression network was constructed containing 40 DEGs and 101 edges. The genes were mainly associated with collagen fibril organization, extracellular matrix organization and translation. Two modules were identified from the gene coexpression network, which were implicated in muscle contraction and extracellular matrix organization, respectively. Several critical genes were disclosed, such as MYH11, COL5A2 and ribosomal proteins. Nine relevant small molecule drugs were identified, such as scriptaid and STOCK1N-35874. Accordingly, a total of 17 TFs and 10 miRNAs related to COAD were acquired, such as ETS2, NFAT, AP4, miR-124A, MiR-9, miR-96 and let-7.

CONCLUSIONS: Several critical genes and relevant drugs, TFs and miRNAs were revealed in COAD. These findings could advance the understanding of the disease and benefit therapy development.

Key Words
Colon adenocarcinoma, RNA-seq data, Differentially expressed genes, Gene coexpression network, Functional enrichment analysis.

Introduction

Colon adenocarcinoma (COAD) is the third most common type of cancer, accounting for about 10% of all cases[1]. Risk factors include smoking, alcohol, older age and inherited genetic disorders.

Mutations that contribute to adenoma formation or progression have been identified in a number of genes. Mutations in the Wnt signaling pathway was reported as the most frequent cause, such as adenomatous polyposis coli (APC). Mutation in APC results in accumulation of β-catenin (CTNNB1) which subsequently activates transcription of proto-oncogenes[2]. Mutation in CTNNB1 itself can also lead to accumulation and tumorigenesis[3]. Moreover, mutations in other genes with a function similar to APC have been reported, such as AXIN1[4], AXIN2[5], TCF7L2[6] and NKD1[7]. Notch signaling cooperates with APC mutation to induce adenoma formation[8,9]. Sikandar et al[10] indicated that Notch signaling is required for formation and self-renewal of

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tumor-initiating cells and repression of secretory cell differentiation in COAD.

Cell cycle-related genes and apoptosis-related genes, such as TP53, transforming growth factor-β (TGF-β)[11] and deleted in colorectal cancer (DCC)[12], were also implicated in the pathogenesis of COAD. It's reported that certain types of p53 mutation are associated with poor prognosis in colon cancer[13]. TGF-β suppresses tumor progression by inhibition of IL-6 trans-signaling[11].

microRNAs (miRNAs) were also considered as critical factors in the pathogenesis of COAD. MiR-21 down-regulates tumor suppressor Pdcd4 and stimulates invasion and metastasis of COAD[14], which also negatively regulates Cdc25A and cell cycle progression in COAD[15]. MiR-34a induces senescence-like growth arrest through modulation of the E2F pathway in human colon cancer cells[16].

Although progress in genes and miRNAs studies deepen our understanding about the molecular pathogenesis mechanisms of colon cancer, there remain difficulties to link the knowledge as a whole, which may help us to find the key to conquer the malignant disease. Gene expression profiling provides a global view of gene expression and enables identification of critical genes in the progression of COAD. In the present study, RNA-seq data of COAD were collected and analyzed with currently available bioinformatics tools to screen out key genes of COAD. Meanwhile, relevant small molecules drugs, transcription factors (TFs) and microRNAs (miRNAs) were also predicted, which might be helpful for future therapy development.

Materials and Methods

Data source

Gene expression data (RNASeqV2) of COAD were downloaded from The Cancer Genome Atlas (TCGA) by TCGA-Assembler. Data normalization was performed with package *TCC*. A total of 437 COAD and 39 control samples were collected.

Screening of DEGs

Differential analysis was performed for the RNA-seq data with package edgeR[17] of *R*. False positive discovery (FDR) correction[18] was applied on *p*-value with package multitest. FDR < 0.05 and |log2 (fold change)|>1 were set as the threshold to screen out DEGs.

Cluster analysis

Bidirectional hierarchical clustering[19] with Euclidean distance was performed using package *pheatmap* for the expression levels of the DEGs.

Construction of gene coexpression network

Correlation between DEGs were calculated with package EBcoexpress[20] of *R*. Relationships with correlation coefficient > 0.6 were retained in the gene coexpression network that was visualized with Cytoscape[21].

Functional enrichment analysis annotation

GO annotation[22] was acquired for each DEG. The number of genes with certain functional terms was calculated with a perl script.

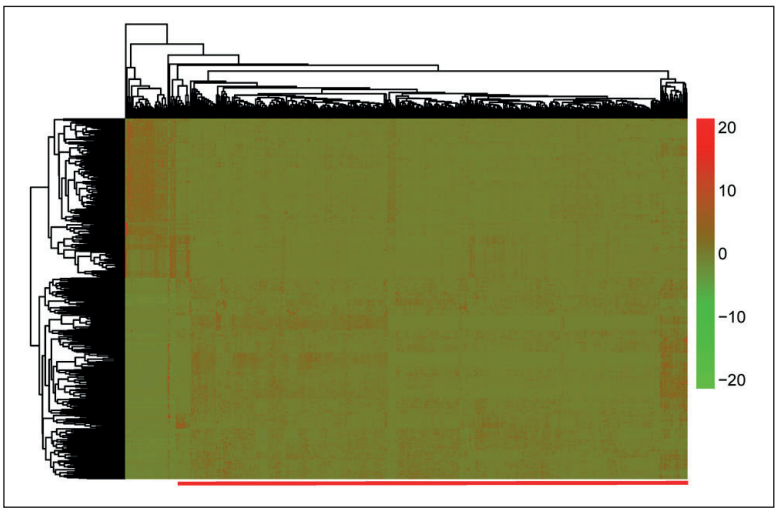

Figure 1. Bidirectional hierarchical clustering result. Bidirectional hierarchical clustering result for the 457 differentially expressed genes and 476 samples. The red line indicates colon adenocarcinoma samples.

GO enrichment analysis was performed for the DEGs in the gene coexpression network with DAVID (Database for Annotation, Visualization and Integration Discovery, http://david.abcc.ncifcrf.gov/)[23]. p-value < 0.05 was set as the threshold.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was also performed for the genes with KOBASS 2.0[24]. p-value < 0.05 was set as the threshold.

Module analysis

Modules were identified with Mcode[25] of Cytoscape[21] (degree cutoff ≥ 2 and k-core ≥ 2). Functional annotations were given for each module with Bingo[26] based on hypergeometric distribution (adjusted p-value < 0.01).

Screening of relevant small molecule drugs, miRNAs and TFs

Relevant small molecules drugs were predicted by Connectivity map (cmap)[27] and those with |score| > 0.8 were retained.

Relevant miRNAs and TFs were searched with WebGestalt[28,29]. Adjusted p-value < 0.05 was set as the threshold.

Results

Differentially expressed genes

A total of 9646 genes were identified from 476 samples, including 437 COAD samples and 39 controls. Differential analysis revealed 457 DEGs, 255 up-regulated genes and 202 down-regulated genes.

Figure 1 showed the result of bidirectional hierarchical clustering for the 457 DEGs and 476 samples. Different gene expression patterns were observed between COAT samples and controls, suggesting the reliability of the DEGs.

GO annotations of the DEGs

Go annotations of the DEGs are shown in Figure 2. Up-regulated genes are shown in red while down-regulated genes are shown in green. Cancer-related biological processes were included, such as death, biological adhesion, immune system process and cellular process.

Gene coexpression network

Relationships with correlation coefficient > 0.6 were retained in the gene coexpression network (Figure 3). A total of 40 DEGs (i.e. nodes) and 101 edges

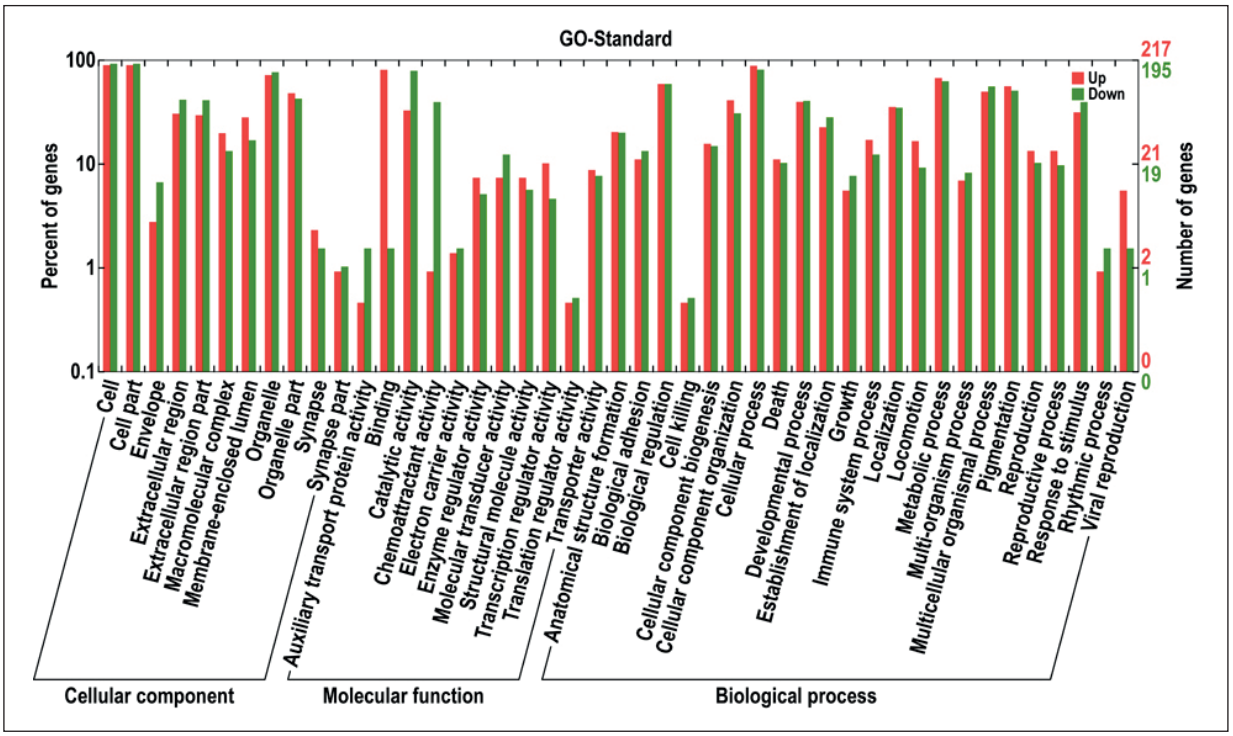

Figure 2. GO annotations for the differentially expressed genes. Up-regulated genes are in red while down-regulated genes are in green. Left vertical axis indicates the percentage of genes while right vertical axis indicates number of genes.

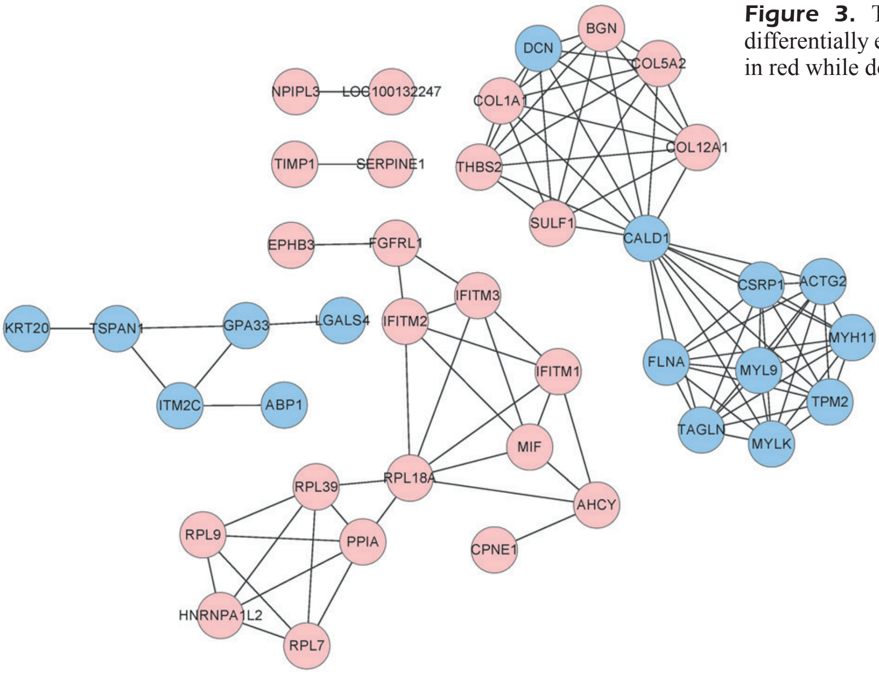
BGN
DCN
COL5A2
NPIPL3
LOC100132247
COL1A1
TIMP1
SERPINE1
THBS2
COL12A1
SULF1
CALD1
EPHB3
FGFRL1
IFITM3
CSRP1
ACTG2
KRT20
TSPAN1
GPA33
LGALS4
IFITM2
MYH11
IFITM1
FLNA
MYL9
ITM2C
ABP1
TPM2
TAGLN
MYLK
MIF
RPL39
RPL18A
AHCY
RPL9
PPIA
CPNE1
HNRNPA1L2
RPL7

Figure 3. The gene coexpression network of differentially expressed genes. Up-regulated genes in red while down-regulated genes are in blue.

were included, including 24 up-regulated genes and 16 down-regulated genes. Hub genes could be identified from the network, such as caldesmon 1 (CALD1), filamin A alpha (FLNA), cysteine and glycine-rich protein 1 (CSRP1), ribosomal protein L18a (RPL18A) and myosin light chain 9 regulatory (MYL9). The relative more correlation of hub genes indicates their important role in the pathogenesis of COAD.

Functional enrichment analysis result

GO enrichment analysis revealed 10 significantly over-represented terms (Figure 4), such as collagen fibril organization, extracellular matrix organization and translation.

KEGG pathway enrichment analysis showed that vascular smooth muscle contraction, focal adhesion, ribosome and ECM-receptor interaction were significantly over-represented (Table I).

Modules and functions

Two modules were identified from the gene coexpression network (Figure 5). Module A included 16 genes, which were implicated in muscle contraction and extracellular matrix organization (Table II). Module B contained 10 up-regulated genes, which were mainly involved in translation (Table II).

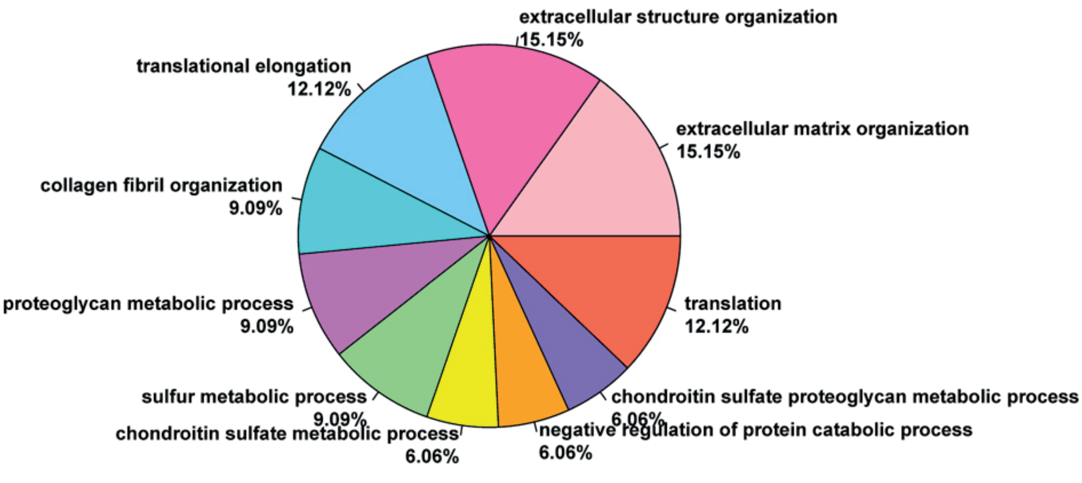
extracellular structure organization
15.15%
translational elongation
12.12%
extracellular matrix organization
15.15%
collagen fibril organization
9.09%
proteoglycan metabolic process
9.09%
translation
12.12%
sulfur metabolic process
9.09%
chondroitin sulfate proteoglycan metabolic process
6.06%
chondroitin sulfate metabolic process
6.06%
negative regulation of protein catabolic process
6.06%

Figure 4. GO terms significantly over-represented in the genes from the coexpression network.

Table I. KEGG pathways over-represented significantly from the gene coexpression network.

ID	Pathway	*p*-value	Genes
hsa04270	Vascular smooth muscle contraction	9.99E-04	ACTG2, CALD1, MYH11, MYLK, MYL9
hsa04510	Focal adhesion	1.11E-03	COL1A1, COL5A2, THBS2, FLNA, MYLK, MYL9
hsa03010	Ribosome	5.15E-03	RPL18A, RPL7, RPL9, RPL39
hsa04512	ECM-receptor interaction	4.62E-02	COL1A1, COL5A2, THBS2

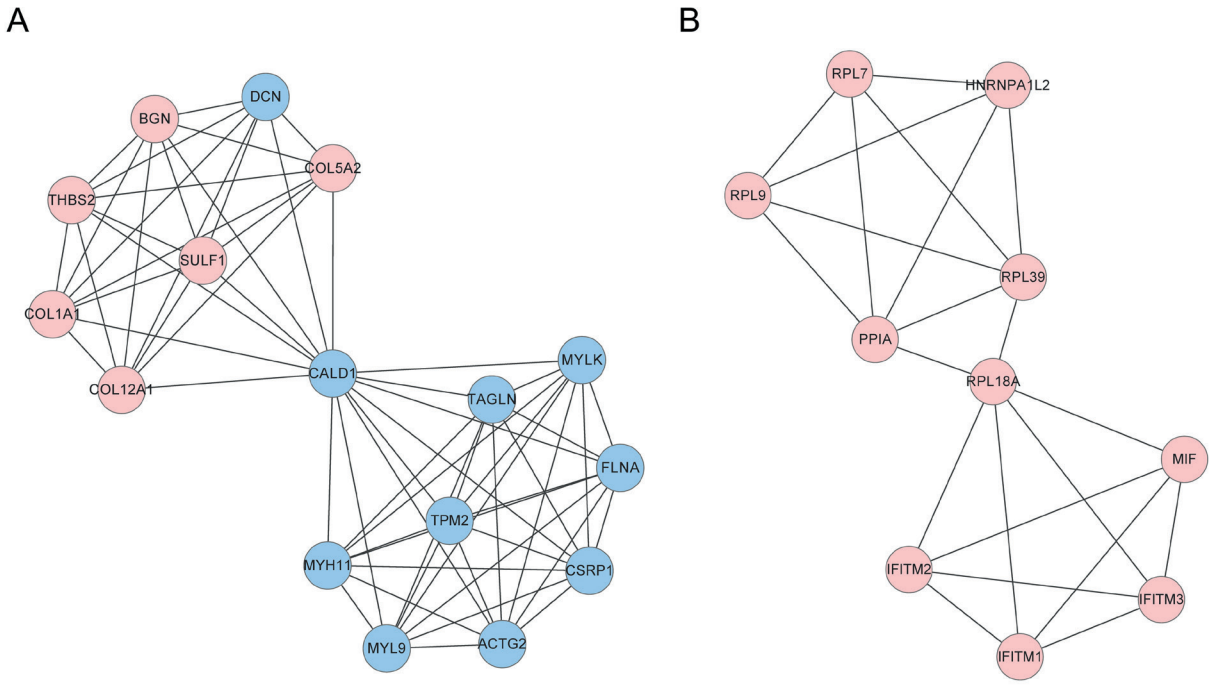

Figure 5. Two modules identified from the gene coexpression network. Up-regulated genes are in red while down-regulated genes are in blue.

Table II. GO functional terms of module A and module B.

ID	*p*-value	Correlation *p*-value	Number of gene	Description
Module A				
6936	6.52E-09	1.92E-06	6	Muscle contraction
3012	1.10E-08	1.92E-06	6	Muscle system process
30198	4.98E-08	5.80E-06	5	Extracellular matrix organization
43062	4.51E-07	3.95E-05	5	Extracellular structure organization
32501	1.12E-04	4.75E-03	12	Multicellular organismal process
3008	4.24E-04	1.14E-02	7	System process
Module B				
6414	4.05E-07	1.21E-04	4	Translational elongation
6412	3.20E-05	4.78E-03	4	Translation

Relevant small molecule drugs, miRNAs and TFs

Nine relevant small molecule drugs were identified (Table III). Scriptaid had the maximum negative correlation coefficient, while STOCK1N-35874 had the maximum positive correlation coefficient.

A total of 17 TFs were also revealed (Table IV), such as v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ETS2), serum response factor (SRF), nuclear factor of activated T cells (NFAT), paired box 4 (PAX4) and transcription factor AP4 (AP4).

Besides, 10 miRNAs were disclosed (Table V), such as miR-124A, miR-9, miR-96 and let-7.

Discussion

In the present study, a total of 457 DEGs were identified from 437 COAD samples and 39 controls. A gene coexpression network containing 40 DEGs and 101 edges were constructed. Functional enrichment analysis showed that they were associated with vascular smooth muscle contraction, focal adhesion, ribosome and ECM-receptor interaction. Two modules were extracted from the network, which were associated with extracellular matrix organization and translation, respectively.

Several DEGs have been involved in the pathogenesis of COAD. The function of myosin heavy chain 11 (MYH11) was considered as a major contractile protein, converting chemical energy into mechanical energy through the hydrolysis of ATP. Alhopuro et al[30] found that MYH11 mutations contribute to human intestinal neoplasia as it may affect the cellular energy balance or disturb cell lineage decisions in tumor progenitor cells. Myosin light chain kinase (MYLK) phosphorylates myosin regulatory light chains to facilitate myosin interaction with actin filaments to produce contractile activity. Han et al[31] reported that a transcribed pseudogene of MYLK (MYLKP1) inhibits MYLK expression in cancer cells by decreasing RNA stability, leading to increased cell proliferation. Fischer et al[32] found that collagen type V alpha 2 (COL5A2) is not expressed in a normal colon but it is co-expressed with COL11A1 in the tumors. Thus, they speculate that COL5A2 is associated with COAD. Thrombospondin 2 (THBS2) expression was correlated with inhibition of angiogenesis and metastasis of colon cancer[33]. Filamin A (FLNA) has been implicated in breast cancer[34] and prostate cancer[35]. Since it participates in remodeling the cytoskeleton to effect changes in cell shape and migration, we speculated that it might be an important gene in the development of COAD. Previous studies have revealed dysregulation of ribosomal proteins in COAD[36,37]. Down-regulation of RPL39 gene has been found in metastatic colorectal carcinoma. Therefore, the exact roles of ribosomal protein L7 (RPL7), RPL9, RPL39 and RPL18A are worthy of further researches.

Relevant small molecule drugs were retrieved. Scriptaid is a histone deacetylase inhibitor and it induces cell cycle arrest and epigenetic change in colon cancer cells[38]. The combination of histone deacetylase inhibitor (scriptaid) and proteasome inhibitors leads to synergistic induction of apoptosis and chemosensitization of human colorectal cancer cells[39].

Relevant TFs were also acquired. ETS proteins are transcription factors that activate or repress the expression of genes that are involved in various biological processes, including cellular proliferation, differentiation, development, transformation and apoptosis[40]. ETS2 plays a role in transcriptional regulation of human telomerase reverse transcriptase, an essential component of telomerase to maintain telomeres and prohibit cell senescence in COAD[41]. NFAT transcription factors were involved in promoting invasion[42]. AP4 was a mediator of epithelial-mesenchymal transition (EMT) that contributes to metastatic processes in COAD[43]. Shi et al[44] further pointed out that p53-induced miR-15a/16-1 and AP4 form a double-negative feedback loop to regulate EMT in COAD.

MiRNAs also played important roles in the progression of various cancers. Relevant miRNAs of DEGs were investigated in the present study, and some of them have been involved in

Table III. 9 relevant small molecule drugs.

cmap Name	Correlation	*p*-value
Scriptaid	-0.970	8.00E-05
Rifabutin	-0.841	8.09E-03
Arachidonic acid	-0.823	1.12E-02
Vorinostat	-0.808	0.00E+00
0297417-0002B	-0.808	1.42E-02
16,16-dimethyl-prostaglandin E2	0.925	8.00E-04
Spaglumic acid	0.938	7.55E-03
AH-6809	0.947	4.99E-03
STOCK1N-35874	0.977	8.70E-04

Table IV. 17 relevant transcription factors.

Transcription factor	ID	Parameters
hsa_GGGAGGRR_V$MAZ_Q6	DB_ID:2430	O=59;aw*p*=1.40E-16;adj*p*=8.82E-15
hsa_TATAAA_V$TATA_01	DB_ID:2456	O=43;raw*p*=6.10E-16;adj*p*=1.92E-14
hsa_GGGCGGR_V$SP1_Q6	DB_ID:2452	O=66;raw*p*=1.44E-15;adj*p*=3.02E-14
hsa_V$SRF_Q5_01	DB_ID:2299	O=19;raw*p*=1.03E-14;adj*p*=1.62E-13
hsa_CAGGTG_V$E12_Q6	DB_ID:2409	O=57;raw*p*=8.03E-14;adj*p*=1.01E-12
hsa_V$SRF_Q4	DB_ID:2286	O=17;raw*p*=1.88E-12;adj*p*=1.91E-11
hsa_TGANTCA_V$AP1_C	DB_ID:2402	O=35;raw*p*=2.12E-12;adj*p*=1.91E-11
hsa_V$SRF_Q6	DB_ID:1975	O=17;raw*p*=7.08E-12;adj*p*=5.58E-11
hsa_CCAWWNAAGG_V$SRF_Q4	DB_ID:2454	O=11;raw*p*=7.94E-11;adj*p*=5.56E-10
hsa_RTAAACA_V$FREAC2_01	DB_ID:2417	O=28;raw*p*=6.86E-10;adj*p*=4.32E-09
hsa_RYTTCCTG_V$ETS2_B	DB_ID:2415	O=28;raw*p*=2.62E-08;adj*p*=1.27E-07
hsa_CAGCTG_V$AP4_Q5	DB_ID:2403	O=34;raw*p*=2.56E-08;adj*p*=1.27E-07
hsa_TGGAAA_V$NFAT_Q4_01	DB_ID:2437	O=36;raw*p*=5.17E-07;adj*p*=1.63E-06
hsa_V$TATA_01	DB_ID:2025	O=11;raw*p*=5.38E-06;adj*p*=1.17E-05
hsa_GGGTGGRR_V$PAX4_03	DB_ID:2445	O=21;raw*p*=0.0010;adj*p*=0.0011
hsa_YTATTTTNR_V$MEF2_02	DB_ID:2431	O=11;raw*p*=0.0191;adj*p*=0.0201
hsa_GTGACGY_V$E4F1_Q6	DB_ID:2412	O=10;raw*p*=0.0292;adj*p*=0.0302

Note: number of genes in the gene set and also in the category (O), *p*-value from hypergeometric test (raw*p*), and *p*-value adjusted by the multiple test adjustment (adj*p*).

COAD. MiR-124A was involved in regulation of colorectal cancer growth via inhibition of the Warburg effect[45]. Deregulation of miR-9 promotes proliferation and tumor cell survival in COAD[46]. MiR-96 was up-regulated in COAD and was correlated with liver metastasis[47]. Besides, let-7 was regarded as a potential growth suppressor in COAD[48]. Researches on other relevant miRNAs might provide therapeutic targets for COAD.

Overall, several critical genes were identified in COAD through a bioinformatics analysis of RNA-seq data, such as MYH11, MYLK, COL5A2 and FLNA. Relevant small molecule drugs, TFs and miRNAs were also revealed and some of them have been implicated in COAD. These findings could improve the understanding about the pathogenesis of the disease and also benefit future therapy development.

Acknowledgements
The authors declare that no conflicts of interest exist.

Conflicts of interest
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Table V. 10 relevant microRNAs.

Transcription factor	ID	Parameters
hsa_TGGTGCT,MIR-29	DB_ID:671	O=17;raw*p*=6.70E-07;adj*p*=7.37E-06
hsa_GTGCCTT,MIR-506	DB_ID:712	O=19;raw*p*=3.45E-06;adj*p*=1.22E-05
hsa_TGCTGCT,MIR-15	DB_ID:666	O=17;raw*p*=4.45E-06;adj*p*=1.22E-05
hsa_TTTGCAC,MIR-19	DB_ID:696	O=16;raw*p*=2.81E-06;adj*p*=1.22E-05
hsa_AATGTGA,MIR-23	DB_ID:683	O=13;raw*p*=2.52E-05;adj*p*=4.62E-05
hsa_TGCCTTA,MIR-124A	DB_ID:811	O=15;raw*p*=2.44E-05;adj*p*=4.62E-05
hsa_ACCAAAG,MIR-9	DB_ID:809	O=14;raw*p*=3.44E-05;adj*p*=5.41E-05
hsa_GTGCCAA,MIR-96	DB_ID:821	O=10;raw*p*=1.00E-04;adj*p*=1.00E-04
hsa_CAGTATT,MIR-200	DB_ID:679	O=12;raw*p*=3.00E-04;adj*p*=3.00E-04
hsa_CTACCTC,LET-7	DB_ID:664	O=10;raw*p*=9.00E-04;adj*p*=9.00E-04

Note: number of genes in the gene set and also in the category (O), *p*-value from hypergeometric test (raw*p*), and *p*-value adjusted by the multiple test adjustment (adj*p*).

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