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Bioinformatic analysis of RNA-seq data unveiled critical genes in rectal adenocarcinoma

Z.-G. ZUO[1], X.-F. ZHANG[2], X.-Z. YE[1], Z.-H. ZHOU[1], X.-B. WU[1], S.-C. NI[1], H.-Y. SONG[1]

[1]Department of Colorectal Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang, P.R. China
[2]Department of Colorectal Surgery, the Third People's Hospital of Hangzhou City, Hangzhou, P.R. China

Zhi-Gui Zuo and Xiu-Feng Zhang are co-first authors

Abstract. – OBJECTIVE: RNA-seq data of rectal adenocarcinoma (READ) were analyzed with bioinformatics tools to unveil potential biomarkers in the disease.

MATERIALS AND METHODS: RNA-seq data of READ were downloaded from The Cancer Genome Atlas (TCGA) database. Differential analysis was performed with package edgeR. False discovery rate (FDR) < 0.05 and |log2 (fold change)|>1 were set as cut-off values to screen out differentially expressed genes (DEGs). Gene coexpression network was constructed with package *Ebcoexpress*. Gene Ontology enrichment analysis was performed for the DEGs in the gene coexpression network with DAVID online tool. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was also performed for the genes with KOBASS 2.0.

RESULTS: A total of 620 DEGs, 389 up-regulated genes, and 231 down-regulated genes, were identified from 163 READ samples and 9 normal controls. A gene coexpression network consisting of 71 DEGs and 253 edges were constructed. Genes were associated with ribosome and focal adhesion functions. Three modules were identified, in which genes were involved in muscle contraction, negative regulation of glial cell proliferation and extracellular matrix organization functions, respectively. Several critical hub genes were disclosed, such as RPS2, MMP1, MMP11 and FAM83H. Thirteen relevant small molecule drugs were identified, such as scriptaid and spaglumic acid. A total of 8 TFs and 5 miRNAs were acquired, such as MYC, NFY, STAT5A, miR-29, miR-200 and miR-19.

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

Key Words:
Rectal adenocarcinoma, Differentially expressed genes, Gene coexpression network, Small molecule drugs, Transcription factors, microRNAs.

Introduction

Colorectal cancer (CRC) is the third most common type of cancer, accounting for 10% of all cases. Rectal adenocarcinoma (READ) is a common type of CRC. Risk factors for CRC include lifestyle, older age, and inherited genetic disorders. Much attention has been paid in identification of markers with diagnostic or prognostic value. For instance, the expression alterations of CXCR5 and CXCL13 are associated with poor prognosis of advanced CRC[1].

At present, various biomarkers have been identified in READ. Reportedly, caudal-related homeobox 2 (CDX2) is a sensitive marker for READ[2]. Moussata et al[3] find that X chromosome-linked inhibitor of apoptosis (XIAP) is a radioresistance factor and prognostic marker for radiotherapy in READ. Hypoxia-inducible factor-1α is also identified as a prognostic marker[4]. Moreover, carcinoembryonic antigen (CEA) is considered as a serum biomarker of disease activity in metastatic READ[5]. Lam et al[6] report that p16 is a marker indicating the aggressiveness and morphological type of colorectal adenocarcinoma. Besides, other biomarkers such as human telomerase reverse transcriptase (HTERT)[7], N-myc downstream regulated 1 (NDRG1)[8] and CD73[9] are also reported. microRNAs (miRNAs) are small non-conding RNAs that have significant roles in gene regulations at transcriptional and post-transcriptional levels. Several miRNAs have been proposed as biomarkers for the prognosis of CRC such as hsa-miR-339-5p, hsa-miR-19a and hsa-miR-29b[10].

Gene expression profiling has been used to identify novel biomarkers[11,12] in READ. Petty et al[13] identify APRIL as a clinical chemo-resistance biomarker in READ through gene expression

Corresponding Author: Shi-Chang Ni, MD; Hua-Yu Song, MD; e-mail: zhshhdgdh@hotmail.com

profiling. Gene expression profiling was also used to investigate the response of READ to radiochemotherapy[14] and recurrence[15]. Liu et al[16] reveal abnormalities in signaling transduction via RNA sequencing. A considerable number of gene expression data have been obtained; however, these data are not fully exploited and need to be re-analyzed, which might provide new insight into READ progression and prognosis.

In the present study, RNA-seq data of READ were collected and analyzed with bioinformatics tools. Differentially expressed genes (DEGs) were identified and then a gene coexpression network was constructed, from which hub genes were revealed. Besides, relevant transcription factors (TFs), microRNAs (miRNAs) and small molecule drugs were also investigated. These findings could advance the knowledge about the pathogenesis of READ and thus benefit therapy development.

Materials and Methods

Gene Expression Data

RNASeqV2 data of READ were downloaded from The Cancer Genome Atlas (TCGA) with TCGA-Aseembler. Data normalization was performed with package TCC[17]. A total of 163 READ samples and 9 normal controls were contained in the dataset.

Screening of DEGs

Differential analysis was performed with package edgeR[18] of R. False discovery rate (FDR) correction[19] was done with package multtest[20]. FDR < 0.05 and $|\log_2(\text{fold change})| > 1$ were set as the cut-off values to screen out DEGs.

Cluster Analysis

Bidirectional hierarchical clustering[21] with Euclidean distance was performed using package pheatmap[22] for the detection of DEGs' expression level. The result was indicated by the heat map.

Construction of Gene Coexpression Network

Correlation between DEGs were calculated with EBcoexpress package[23] of R. Interacted genes with correlation coefficient > 0.6 were retained in the gene coexpression network, which was visualized by Cytoscape software[24].

Functional Enrichment Analysis

Gene Ontology (GO)[25] enrichment analysis was performed for the DEGs in the gene coexpression network using DAVID (Database for Annotation, Visualization and Integration Discovery, http://david.abcc.ncifcrf.gov/) database[26]. *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[27]. *p*-value < 0.05 was set as the threshold.

Module Analysis

Modules were identified with Mcode[28] of Cytoscape[24], with the criteria of degree cutoff ≥ 2 and k-core ≥ 2. Functional annotations were conducted for each module with Bingo[29] based on hypergeometric distribution (adjusted *p*-value < 0.01).

Screening of Relevant Small Molecule drugs, miRNAs and TFs

Relevant small molecule drugs were predicted by the Connectivity map (cmap) tool[30], and those with |score| > 0.8 were retained.

Relevant miRNAs and TFs were searched using WebGestalt webserver[31, 32]. Adjusted p-value < 0.05 was set as the threshold.

Results

DEGs Between READ Samples and Controls

A total of 9,979 genes were identified from 172 samples, including 163 READ samples and 9 controls. Differential analysis unveiled 620 DEGs, including 389 up-regulated genes and 231 down-regulated genes.

Figure 1 is the result of bidirectional hierarchical clustering for the 620 DEGs and 172 samples. Different gene expression patterns were observed between READ samples and controls, suggesting the DEGs could well distinguish the two kinds of samples.

GO Annotations of the DEGs

GO annotations of the DEGs are shown in Figure 2. Cancer-related biological processes were included, such as death, biological adhesion, immune system process and growth.

Gene Coexpression Network

Interplayed genes with correlation coefficient > 0.6 were retained in the gene coexpression network (Figure 3). A total of 71 DEGs (i.e. nodes) and 253 edges (lines between these nodes) were included, involving 45 up-regulated genes

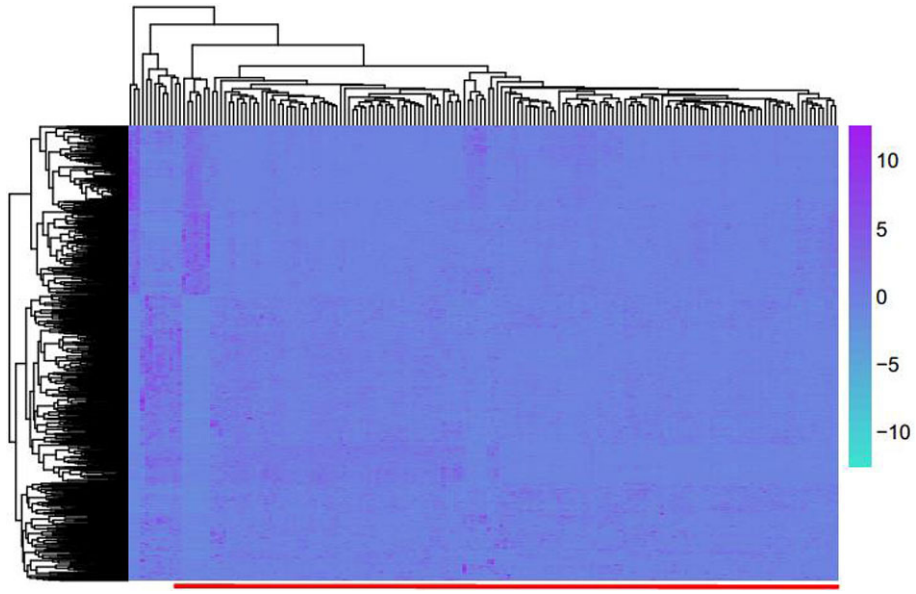

Figure 1. Bidirectional hierarchical clustering result for the 620 differentially expressed genes and 172 samples. Red line indicates disease samples.

and 26 down-regulated genes. The numbers of interacted genes of a node is defined as the degree of this node. Hub genes (the nodes with degrees ≥ 8) were highlighted, such as ribosomal protein S2 (RPS2), myosin light chain kinase (MYLK), thrombospondin 2 (THBS2), tensin 1 (TNS1), glutamate receptor ionotropic N-methyl D-aspartate-associated protein 1 (GRINA) and carcinoembryonic antigen-related cell adhesion molecule 7 (CEACAM7).

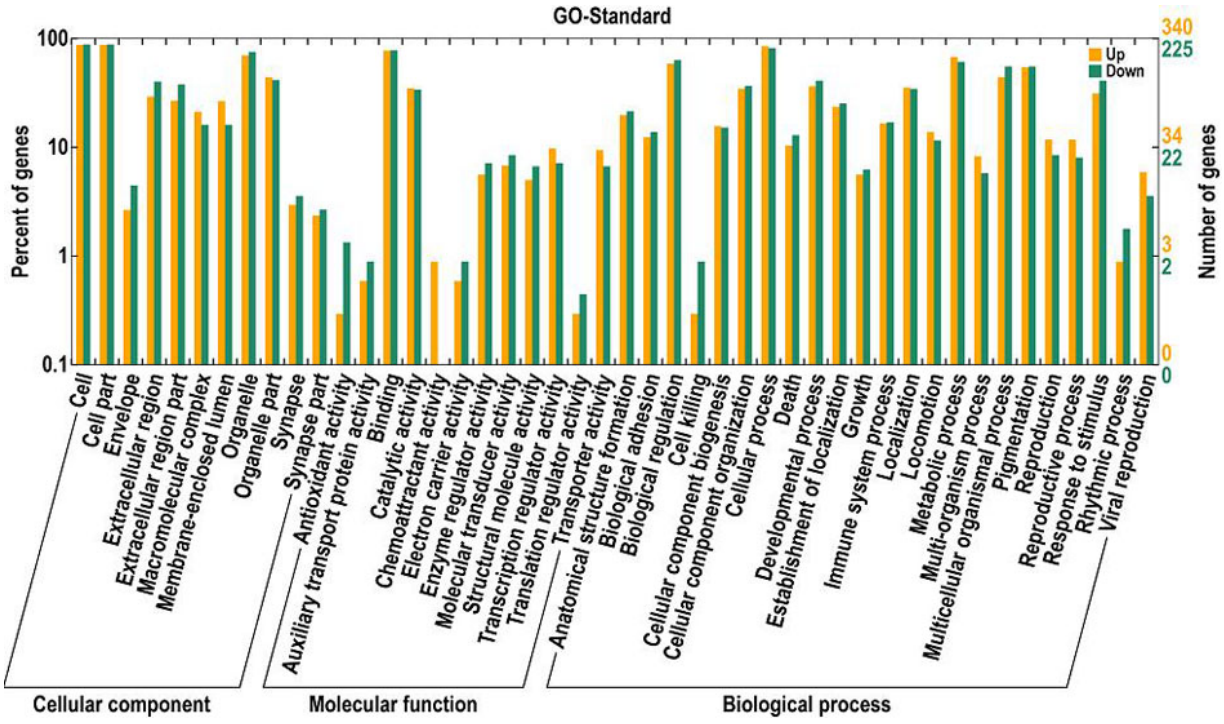

Figure 2. Gene Ontology annotations for the differentially expressed genes. Up-regulated genes are in orange while down-regulated genes are in green. Left vertical axis indicates percentage of genes while right vertical axis indicates number of genes.

Functional Enrichment Analysis of the Coexpressed Genes

GO enrichment analysis revealed 10 significantly over-represented terms for genes in the coexpression network (Figure 4), such as translational elongation, response to oxidative stress, response to cAMP and multicellular organismal metabolic process.

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

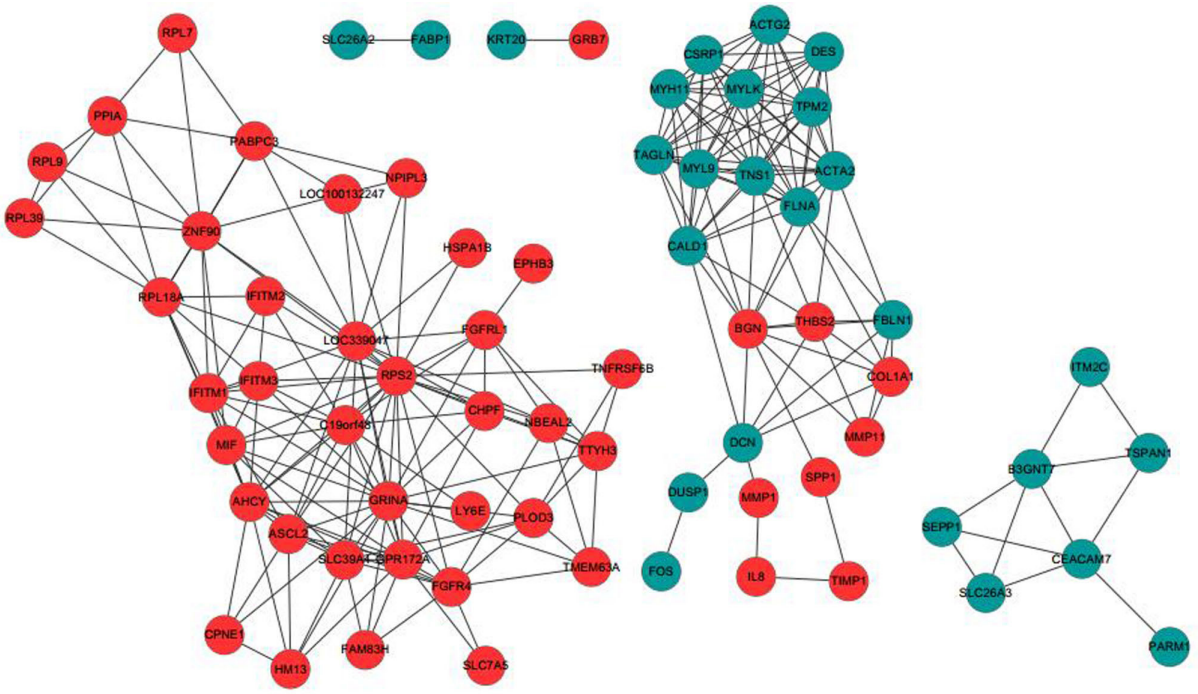

Figure 3. The gene coexpression network of differentially expressed genes. Up-regulated genes in red while down-regulated genes are in green.

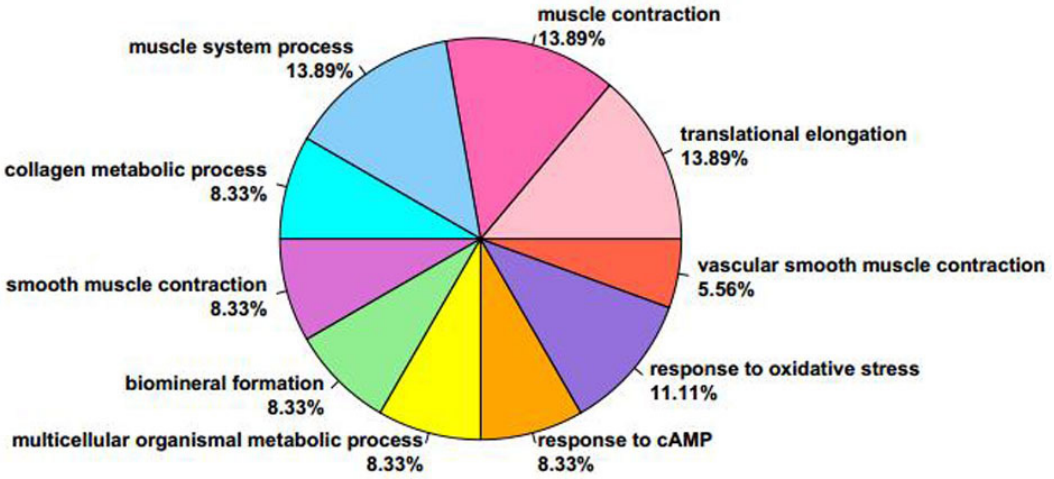

Figure 4. Gene Ontology terms significantly over-represented in the genes from the gene coexpression network.

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

Term	Count	*p*-value
hsa04270:Vascular smooth muscle contraction	6	5.94E-04
hsa03010:Ribosome	5	0.0019944
hsa04510:Focal adhesion	6	0.0077388

KEGG: Kyoto Encyclopedia of Genes and Genomes.

Table II. Functional terms of the three modules.

GO-ID	*p*-value	Corr *p*-value	No. of gene	Description
Module A				
32501	1.69E-04	6.69E-03	9	multicellular organismal process
6936	6.64E-15	1.55E-12	8	muscle contraction
3012	1.35E-14	1.58E-12	8	muscle system process
3008	5.49E-07	4.28E-05	8	system process
7010	1.71E-04	6.69E-03	4	cytoskeleton organization
30036	4.82E-04	1.61E-02	3	actin cytoskeleton organization
30029	5.78E-04	1.69E-02	3	actin filament-based process
6939	1.65E-04	6.69E-03	2	smooth muscle contraction
7015	1.25E-03	2.93E-02	2	actin filament organization
Module B				
60253	1.26E-03	4.56E-02	1	negative regulation of glial cell proliferation
60251	1.26E-03	4.56E-02	1	regulation of glial cell proliferation
10624	1.26E-03	4.56E-02	1	regulation of Schwann cell proliferation
10626	1.26E-03	4.56E-02	1	negative regulation of Schwann cell proliferation
Module C				
32963	2.54E-05	2.38E-03	2	collagen metabolic process
44259	3.09E-05	2.38E-03	2	multicellular organismal macromolecule metabolic process
44236	4.33E-05	2.38E-03	2	multicellular organismal metabolic process
9612	1.03E-04	4.26E-03	2	response to mechanical stimulus
30198	3.05E-04	1.01E-02	2	extracellular matrix organization
43062	7.35E-04	1.73E-02	2	extracellular structure organization
9628	4.60E-03	3.40E-02	2	response to abiotic stimulus
9605	8.44E-03	3.66E-02	2	response to external stimulus
9887	1.11E-02	4.27E-02	2	organ morphogenesis

GO: Gene Ontology.

Modules and Functions

Three modules were identified from the gene coexpression network (Figure 5). Module A included 12 down-regulated genes, which were implicated in muscle contraction and cytoskeleton organization (Table II). Module B contained 11 up-regulated genes, which were involved in regulation of glial cell proliferation (Table II). Module C consisted of 2 up-regulated genes and 2 down-regulated genes, which were related to extracellular matrix organization (Table II).

Relevant Small Molecule Drugs, miRNAs and TFs

Thirteen relevant small molecule drugs were identified (Table III). Scriptaid had the maximum negative correlation coefficient while spaglumic acid had the maximum positive correlation coefficient.

A total of 8 TFs were also revealed (Table IV), such as signal transducer and activator of transcription 5A (STAT5A), nuclear transcription factor Y (NFY) and v-myc avian myelocytomatosis viral oncogene homolog (MYC).

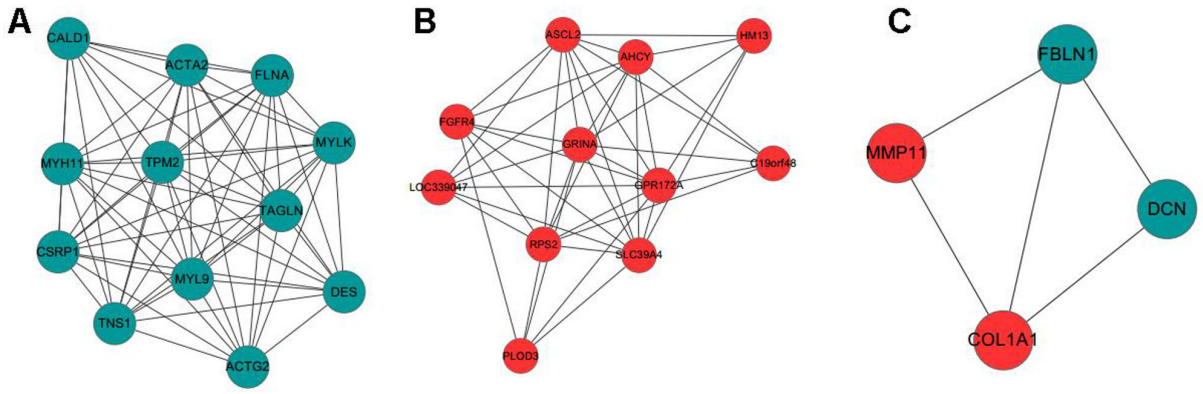

Figure 5. Three modules identified from the gene coexpression network. Up-regulated genes are in red while down-regulated genes are in green.

Besides, 5 miRNAs were selected (Table V), including miR-29, miR-200 and miR-19.

Discussion

In the present study, a total of 620 DEGs were identified from 163 READ samples and 9 normal controls. A gene coexpression network consisting of 71 DEGs and 253 edges were constructed. Functional enrichment analysis showed that they were associated with vascular smooth muscle contraction, ribosome and focal adhesion. Three modules were extracted from the network, which was associated with muscle contraction and cytoskeleton organization, regulation of glial cell proliferation, and extracellular matrix organization, respectively.

Some DEGs have been implicated in CRC or READ. Ribosomal protein S2 (RPS2) is found to be up-regulated in READ and have been suggested as a potential biomarker[33]. Reportedly, actin gamma 2 (ACTG2) is positively correlated with chemotherapy sensitivity of READ[34]. Myosin expression in CRC is associated with tumor progression and metastasis development[35]. Further-

Table III. Thirteen relevant small molecule drugs.

cmap name	Correlation	*p*-value
scriptaid	-0.981	0.00004
rifabutin	-0.935	0.00042
0297417-0002B	-0.928	0.00062
vorinostat	-0.917	0
mycophenolic acid	-0.846	0.00727
meteneprost	0.8	0.003
betulin	0.81	0.01388
6-bromoindirubin-3'-oxime	0.834	0
AH-6809	0.861	0.03927
5248896	0.887	0.0263
xamoterol	0.918	0.00116
STOCK1N-35874	0.972	0.00125
spaglumic acid	0.977	0.00087

cmap: Connectivity map.

Table IV. Eight relevant transcription factors.

Transcription factor	ID	Parameters
hsa_CACGTG_V$MYC_Q2	DB_ID:2434	O=37;raw*p*=3.11e-11;adjp=2.86e-10
hsa_V$TATA_01	DB_ID:2025	O=18;raw*p*=1.82e-10;adjp=1.59e-09
hsa_GATTGGY_V$NFY_Q6_01	DB_ID:2440	O=38;raw*p*=2.20e-10;adjp=1.84e-09
hsa_V$ATF_01	DB_ID:1856	O=17;raw*p*=1.48e-09;adjp=1.05e-08
hsa_V$CDC5_01	DB_ID:2132	O=15;raw*p*=4.26e-08;adjp=2.18e-07
hsa_TAATTA_V$CHX10_01	DB_ID:2408	O=27;raw*p*=6.92e-08;adjp=3.44e-07
hsa_CTAWWWATA_V$RSRFC4_Q2	DB_ID:2448	O=17;raw*p*=2.03e-07;adjp=9.07e-07
hsa_V$STAT5A_02	DB_ID:2114	O=11;raw*p*=2.19e-07;adjp=9.37e-07

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

Table V. Five relevant microRNAs.

MicroRNA	ID	Parameters
hsa_TGGTGCT,MIR-29	DB_ID:671	O=26;raw*p*=3.19e-11;adjp=4.94e-10
hsa_TGCTGCT,MIR-15	DB_ID:666	O=28;raw*p*=2.62e-11;adjp=4.94e-10
hsa_CAGTATT,MIR-200	DB_ID:679	O=23;raw*p*=6.60e-10;adjp=6.82e-09
hsa_GTGCCTT,MIR-506	DB_ID:712	O=26;raw*p*=2.79e-08;adjp=2.16e-07
hsa_TTTGCAC,MIR-19	DB_ID:696	O=21;aw*p*=8.82e-08;adjp=5.47e-07

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

more, the down-regulated myosin heavy polypeptide 11 (MYH11) expression is correlated with poor prognosis in stage II and III CRC[36]. Matrix metallopeptidase 1 (MMP1) polymorphism has been associated with CRC risk[37-39]. Additionally, MMP11, the target of let-7c, is involved in metastasis of CRC[40]. Family with sequence similarity 83, member H (FAM83H) plays an important role in the structural development. Kuga et al[41] report that keratin cytoskeleton organization is regulated by FAM83H-mediated recruitment of casein kinase Iα (CK-1α) to keratins in CRC, and that keratin filament disassembly caused by overexpression of FAM83H and aberrant localization of CK-1α can contribute to the progression of CRC. Dual specificity phosphatase 1 (DUSP1) is a marker of MEK/Erk activation in primary CRC[42].

In addition, relevant small molecule drugs, TFs and miRNAs were also implicated with CRC (or READ) development. Scriptaid combined with proteasome inhibitors has been used to induce apoptosis and chemosensitization of human CRC cells[43]. MYC is overexpressed in CRC[44,45]. Overexpression of NFY-C, a subunit of the transcription factor NFY, is found in READ[46]. NFY-C mRNA levels are highly correlated with time to disease progression, while NFY-C protein expression is significantly higher in metastatic disease[46]. Du et al[47] find that STAT5 isoforms regulate CRC cell apoptosis via reduction of mitochondrial membrane potential and generation of reactive oxygen species. The study by Hong et al[48] shows that inhibition of signal transducer and activator of transcription 5A (STAT5A) promotes apoptosis of CRC cells induced by chemotherapy drugs. Circulating miR-29 is regarded as a promising biomarker in stage III CRC[49]. It's involved in suppression of CRC cell growth and metastasis by repressing insulin-like growth factor 1 (IGF1)[50] and downregulating Tiam1 expression[51]. Moreover, miR-19-mediated inhibition of transglutaminase 2 leads to enhanced invasion and metastasis in CRC[52]; miR-200c inhibits invasion and migration in human CRC cells[53]. Serum miR-200c is considered as a prognostic and metastasis-predictive biomarker in patients with CRC[54].

Conclusions

A number of DEGs were identified in READ and some of them have been implicated in the disease. Further researches on these genes might discover novel biomarkers for diagnosis or prognosis of READ. Besides, relevant small molecule drugs, TFs and miRNAs were discussed. These findings could provide new clues for therapy development of READ.

Acknowledgement

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Conflicts of interest

The authors declare no conflicts of interest.

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