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Research Article

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Identification of Potential Regulatory Network in Colorectal with Liver Metastasis

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Abstract

Background

Liver metastasis is an important cause of death in patients with colorectal cancer(CRC). Increasing evidences indicates that microRNAs (miRNAs) are involved in the pathogenesis of colorectal with liver metastasis(CRLM). This study aims to explore potential miRNA–mRNA regulatory network.

Methods

The GEO database download the microarray datasets GSE56350 and GSE73178. GEO2R was used to conduct differentially expressed miRNAs (DEMs) between CRC and CRLM. The common miRNAs appeared in the two datasets. The transcription factors were predicted by FunRich and miRNet. Then, GO and KEGG pathway analysis for differentially expressed genes (DEGs) performed via DAVID. Besides, these hub genes network was used the STRING and identified by Cytoscape. Finally, the expression of the hub gene expression was used to assess by the GSE81558.

Results

Four DEMs were selected in the datasets. The SP1 was likely to adjust the up-regulated DEMs, however, the YY1 could regulate both up-regulated and down-regulated DEMs. The total of the 3925 genes were predicted(3447 up-regulated DEM genes and 478 down-regulated DEM genes). These predicted genes were mainly linked to Platinum drug resistance, Cellular senescence and ErbB signaling pathway. Through the gene network construction, most of the hub genes were modulated by hsa-miR-204-5p, hsa-miR-122-5p, hsa-miR-95-3p and hsa-miR-552-3p. Among the top 20 hub genes, the expression of CREB1, RHOA and EGFR was significantly different in the GSE81558 dataset.

Conclusion

In this study, miRNA-mRNA in CRLM were screened between CRC patients and CRLM patients to provide a new method to predict for the pathogenesis and development of CRC.

Keywords: microRNAs (miRNAs), colorectal with liver metastasis (CRLM), bioinformatics analysis, miRNA–mRNA regulatory network

Introduction

Colorectal cancer(CRC) is a common malignant tumor that seriously harms human health[1, 2]. Metastatic is the major contributor to mortality in patients with CRC, especially liver metastasis has been shown as one of the leading causes of death in patients with CRC[3, 4]. Despite advances in hepatectomy and adjuvant therapy, the 5-year survival rate for colorectal cancer liver metastases is still only 25-50%[5]. Hence, it is preferred to study the molecular mechanism regulating CRLM, providing evidence for the prevention to improve prognosis of patients.

MicroRNAs (miRNAs) is a class of non-coding RNA composed of 20-24 nucleotides. It specifically binds to the 3' untranslated regions of target genes through the principle of base complementary pairing, blocks the transcription of mRNA, and then inhibits protein synthesis, thus participating in the regulation of biological functions of target genes[6, 7]. More and more studies believe that miRNA is closely related to tumor regulation, including CRLM [8-10]. MiR-623 inhibited interleukin-8 (IL-8) -induced epithelial interstitial transformation of pancreatic cancer cells by inhibiting extracellular regulatory protein kinase (ERK) phosphorylation, demonstrating the important role of MiR-623 in inhibiting in vitro migration and invasion of pancreatic cancer cells and in vivo metastasis[11].

MiR-135a-5p is highly expressed in lung cancer cells, and inhibition of mir-135A-5p in vitro can inhibit cell growth and promote cell apoptosis. Mechanism studies have found that miR-135A-5p promotes the occurrence of lung cancer by targeting LOXL4 gene[12]. Besides, it can also promote the development of cervical cancer by regulating SIAH1/ β -catenin signaling pathway[13].

In this study, we screened DEMs in CRLM compared to CRC without liver metastasis by analyzing two datasets (GSE5635 and GSE73178) from the GEO database. We verified the DEMs, identified the differential expression profile of miRNA with a gradually increasing trend in transcription factor-DEM-target gene and analyzed the these target genes and hub gene network. the risk of colorectal cancer in combination with the demographic data and clinicopathological characteristics of patients with colorectal cancer and colorectal adenoma. Besides, the expression of hub genes in combination with GSE81558 dataset were further confirmed and construct a relationship with miRNA–mRNA to provide the diagnosis and treatment of CRLM.

Methods

MiRNA microarray and DEGs identification

GEO[14] (<http://www.ncbi.nlm.nih.gov/geo>) is an international public functional dataset including high-throughput microarray and sequence-based data. The miRNA expression profiles of GSE56350 and GSE73178 screened the miRNA datasets between CRC and CRLM. The detailed dataset information is shown in Table 1. DEMs between CRC and CRLM across different GEO datasets. $|\log_2FC| > 1$ and an P value < 0.05 are considered significantly. Then, the overlap of DEMs in the two datasets identified by Venn diagram(GSE56350 and GSE73178). All methods were carried out in accordance with relevant guidelines and regulations.

Table 1

Details for GEO Dataset				
GEO Dataset	Sample	CRC sample	CRLM sample	Gene/microRNA
GSE536350	Tumor	46	15	microRNA
GSE73178	Tumor	2	2	microRNA
GSE81558	Tumor	23	19	gene

Forecast of transcription factors(TF)

FunRich (<http://www.funrich.org/>) is a tool for functional enrichment analysis and interactive network analysis with more than 1.5 million annotation files[15], which can predict the upstream TF of DEMs.

Forecast of target genes

miRNet(<https://www.mirnet.ca/>) analyzes the miRNA-target interactions and displaying correlations in the network to predict downstream target genes of DEMs.

GO and KEGG analysis

The Database for Annotation, Visualization and Integrated Discovery (DAVID) is an online bioinformatics tool that can provide GO and KEGG pathway enrichment analysis[16-18]. Through DAVID, we discovered the unique biology of common DEGs and analyze the DEGs. ($P < 0.05$).

Construction of protein-protein interaction (PPI) and miRNA-hubgenes network

The protein interaction network of target genes was constructed using STRING and Cytoscape tools with version of the 3.7.2. Hub genes were considered to be those with the top 30 nodes using the Maximal Clique Centrality (MCC) method. The miRNA-hubgenes network was constructed by the Cytoscape software.

Evaluation of hubgenes by GSE81558 Dataset

Since there is no other data on mRNA expression between CRC and CRLM, we chose GSE81558 in the GEO database to analyze the expression of the hub gene. The dataset analyzes gene expression data in which 23 cases of CRC and 19 cases of CRLM were selected. The Student's t test was used to identify the hub genes between CRC and CRLM.

Results

Identification of DEMs in CRC and CRLM

As mentioned above, we found 32 DEMs (17 up-regulated and 15 down-regulated) were identified in GSE56350, 190 DEMs (84 up-regulated and 106 down-regulated) were identified in the GSE73178 dataset. Through the presented in the heatmaps of DEMs in Figure 1A–B, the specific information is tabled in Supplementary S1–2. Between two DEMs, two up-regulated (hsa-miR-204-5p and hsa-miR-122-5p) and two down-regulated (hsa-miR-95-3p and hsa-miR-552-3p) DEMs were screened out the datasets (Figure 1C-E)

Figure 1 Identification of the potential DEMs.

(A) GSE56350 dataset; (B) GSE73178 dataset; (C) Venn diagram for up-regulated DEMs; (D) for down-regulated DEMs; (E) heatmap of the common DEMs.

Forecast of upstream TF of DEMs

The candidate DEMs were used to predict the upstream TF by the FunRich software and the top five TF for up-regulated and down-regulated DEMs are displayed(Figure 2A and B). For up-regulated DEMs, the TF were SP1,YY1,SP4, EGR1, ARID3A and NKX6-1. For down-regulated DEMs, the TF were YY1 and E2F1.

Figure 2 FunRich predict TF of DEMs.

(A) top five TF of up-regulated DEMs; (B) top five TF of down-regulated DEMs

Target prediction and analysis of downstream genes of DEMs

The miRNet database predicts a total of 3925 target genes for candidate DEMs, among which 3447 target genes are up-regulated and 478 target genes are down-regulated. In order to better visualization, the DEMs and its target genes are depicted in Figure 3A and B. In order to be more direct , we counted the genes shown in Table 2 and plotted target genes presented (Supplementary Table S3).

Table 2

Potential Genes of the Up-regulated and Down-regulated DEMs			
Up-regulated miRNA	Number	Down-regulated miRNA	Number
hsa-mir-204-5p	985	hsa-mir-552-3p	291
hsa-mir-122-5p	2462	hsa-mir-95-3p	187
Total	3447	Total	478

Figure 3 The predicted potential target genes by miRNet.

(A) for up-regulated DEMs-gene; (B) for down-regulated DEMs-gene.

GO and KEGG Analysis

Then, the 3925 target genes were used for GO analysis and KEGG pathway enrichment analysis. For GO analysis, considering biological process (BP), up-regulated genes were enriched in positive regulation of chromosome organization, covalent chromatin modification, histone modification and peptidyl-lysine modification; regarding to up-regulated DEM target genes cellular component (CC) concentrated in focal adhesion, cell-substrate junction and cell-substrate adherens junction; in terms of the molecular function (MF) analysis demonstrated that up-regulated DEM target genes were significantly concentrated in cadherin binding, cell adhesion molecule binding and DNA-binding transcription activator activity.

Furthermore, up-regulated genes pathway analysis indicated that the main enriched in Platinum drug resistance, Cellular senescence, AGE-RAGE signaling pathway in diabetic complications, Chronic myeloid leukemia, Colorectal cancer and Proteoglycans in cancer (Figure 4C). Moreover, for down-regulated genes were significantly HIF-1 signaling pathway, ErbB signaling pathway and Protein processing in endoplasmic reticulum (Figure 4D).

Figure 4 GO and KEGG pathway analysis

GO analysis of (A) up-regulated DEMs ;(B)down-regulated genes;

KEGG pathway analysis of (C) up-regulated DEMs; (D) down-regulated genes

Construction of PPI and DEMs-hubgenes network

In order to better confirm the relationship between the hubgenes of DEMs, we established PPI network through the STRING database. At the same time, we uploaded

the above-mentioned PPI network and imported in Cytoscape. The top 30 hubgenes up-regulated and down-regulated were screened out in Figure 5A and B. The top 10 hubgenes were MMP9, EGFR, NOTCH1, IL1B, RHOA, HSP90AA1, CDH1, IGF1R, SMAD4 and CDC42 in the up-regulated DEMs; the top 10 hubgenes were CCND1, MTOR, HSPA4, FOS, MCL1, ERBB2, CDKN1A, CREB1, MAPK1 and AR in the down-regulated DEMs (Table 3).

Table 3

Top 10 Hubgenes of the significantly up-regulated and down-regulated DEMs by MCC

Up-regulated		Down-regulated	
Gene	Score	Gene	Score
MMP9	3.29E+09	CCND1	6732
EGFR	3.28E+09	MTOR	6489
NOTCH1	3.17E+09	HSPA4	6422
IL1B	3.04E+09	FOS	6047
RHOA	2.89E+09	MCL1	3675
HSP90AA1	2.70E+09	ERBB2	3510
CDH1	2.64E+09	CDKN1A	3420
IGF1R	2.47E+09	CREB1	3335
SMAD4	1.97E+09	MAPK1	3288
CDC42	1.93E+09	AR	3029

Figure 5 The top 30 hubgenes in PPI network of hubgenes.
(A)for up-regulated DEMs-hub genes; (B) for down-regulated DEMs-hub genes.

Based on the bioinformatics analysis, these DEMs and genes was established by Cytoscape software. We constructed regulatory network between up-regulated DEMs and hubgenes were as follows: hsa-mir-204-5p interacted

with seven hub genes, including *MMP9*, *NOTCH1*, *IL1B*, *HSP90AA1*, *CDH1*, *SMAD4* and *CDC42*; hsa-mir-122-5p interacted with six hubgenes, including *EGFR*, *NOTCH1*, *RHOA*, *CDH1*, *IGF1R* and *SMAD4*; The correlation between DEMs of down-regulated and hubgenes were as follows: hsa-mir-552-3p linked with six hub genes, including *HSPA4*, *FOS*, *MCL1*, *ERBB2*, *MAPK1* and *AR*; hsa-mir-95-3p associated with *CCND1*, *MTOR*, *CDKN1A* and *CREB1*.(Figure 6).

Figure 6 Identified potential miRNA–hubgenes network.

Identification of hubgenes expression

To confirm these hubgenes (*MMP9*, *EGFR*, *NOTCH1*, *IL1B*, *RHOA*, *HSP90AA1*, *CDH1*, *IGF1R*, *SMAD4*, *CDC42*, *CCND1*, *MTOR*, *HSPA4*, *FOS*, *MCL1*, *ERBB2*, *CDKN1A*, *CREB1*, *MAPK1* and *AR*), were then identified by the GSE81558 dataset. For these hubgenes, compared with CRC the expressions of *CREB1* and *RHOA* were decreased in CRLM, however, only *EGFR* expression was increased in CRLM(Figure 7A – T).

Figure 7 The expression of the top 20 target genes in primary tumor compared to liver metastasis of colorectal tumor.

(A)AR; (B) CCND1; (C) CDC42; (D) CDH1; (E)CDKN1A; (F) CREB1; (G) EGFR; (H)ERBB2; (I) HSPA4; (J) IGF1R; (K) HSP90AA1; (L) FOS; (M) IL1B; (N) MCL1; (O) MAPK1; (P) MMP9; (Q) MTOR; (R) NOTCH1; (S) RHOA; (T) SMAD4

DISCUSSION

In recently years, increasing studies have been reported in CRLM. However, the prognosis of the CRC patients is still poor because of the appearance of the liver metastasis. Recently, with the development of the microarray technology, the progression of various diseases begin to be discovered due to the genetic alterations. In the study, the GSE56350 and GSE73178 were used to identify DEMs between primary colorectal tumor and colorectal liver metastasis. Two upregulated DEMs (hsa-mir-204-5p and hsa-mir-122-5p) and two downregulated DEMs (hsa-mir-552-3p and hsa-mir-95-3p), which were significantly changed in the datasets, were selected as candidate DEMs. Besides, the up-regulated DEMs found hsa-mir-204-5p could effective restrain cancer cell proliferation[19]. Besides, mir-204-5p can inhibit cell proliferation, promote apoptosis and enhance drug sensitivity by down-regulating RAB22A expression in CRC[20]. The literature reports the miR-122-5p regulated CDC25A expression in CRC cells [21]. For

down-regulated DEMs, miR-552-3p is highly expressed in various types of tumor cells and can be used as a specific molecular marker, especially in CRC. A large number of bioinformatics studies also show that miR-552-3p can be used as a biomarker for diagnosis and treatment of CRC[22]. Using RNA sequencing analysis (RNA-seq), miR-95-3p is linked to cisplatin resistance in gastric cancer, increasing the PI3K/Akt pathway[23, 24]. Notably, to better understand the mechanisms of these miRNAs involved, we need further studies for their roles in CRLM.

MiRNA expression is abnormal in almost all malignant tumors, which act on oncogenes or tumor suppressor genes and regulate by transcription factors[25-27]. SP1 has been documented in CRC[28-30]. The ADEM10, EPHB2, HDAC4, and SEPP1 in CRC through the SP1 inhibit cell migration, invasion, tumor growth and liver metastasis [30]. YY1 regulated the expressions of both up-regulated and down-regulated DEMs. In the study, YY1 as a member of the PcG protein family can be widely expressed in a variety of tissues and cells, which is involved in cell tissue differentiation, chromatin remodeling, and tumor genesis and progression[31-34]. YY1 is closely associated with tumor including metastatic breast cancer[35, 36], colon cancer[37], gastric cancer[38] and prostate cancer[39]. In the CRC, through the NF- κ B/YY1 axis, LINC01578 enhanced its promoter activity.

Triptolide regulates E2F activity by potentially induce G1 cell cycle[40]. In fact, despite of SP4, EGR1, ARID3A and NKX6-1 the remaining transcription factors have been reported in colorectal cancer[41-45], which supports the importance of these candidate DEMs in the mechanism of CRC tumor.

GO and KEGG pathway enrichment analysis was conducted via DAVID. GO analysis revealed that these DEGs were particularly enriched in positive regulation of chromosome organization, covalent chromatin modification, histone modification, peptidyl-lysine modification and regulation of chromosome organization. For the molecular function, these genes were also significantly enriched in cadherin binding, cell adhesion molecule binding, DNA-binding transcription activator activity, RNA polymerase II-specific, protein serine/threonine kinase activity and enhancer binding.

KEGG analysis showed that the hubgenes were mainly focus on the Platinum drug resistance, Cellular senescence, AGE-RAGE signaling pathway in diabetic complications, HIF-1 signaling pathway, ErbB signaling pathway and Protein processing in endoplasmic reticulum.

In the previous studies, drug resistance has been documented to have an association with liver metastasis of CRC. A study showed that the lack of E-cadherin promoted CRC cell growth, invasion and drug resistance, contributing to CRC progression and metastatic dissemination[46]. Besides, Cellular senescence is also an important factor in tumor metastasis. Cancer stem cells could change their phenotypic and functional appearance. These changes are caused by chemotherapy and radiation, leading to changes in the tumor microenvironment [47]. Research reports that HIF-1 α (hypoxia inducible factor-1 α) expression in liver metastasis determines poor prognosis of CRC liver metastasis patients[48]. Therefore, drug resistance, Cellular senescence and HIF-1 signaling pathway may represent novel therapeutic strategies for patients with CRC liver metastasis. However, many of the target genes were down-regulated without difference in GO

analysis . This may hint that upregulated DEMs may play a more critical role in the liver metastasis of CRC.

In order to screen the DEM-hubgenes of CRLM, we found that these genes could be potentially targeted by hsa-miR-204-5p, hsa-miR-122-5p, hsa-miR-95-3p and hsa-miR-552-3p. Among the top 20 hub genes, three genes (CREB1, RHOA and EGFR) expressions were significantly different in the GSE81558 dataset. CREB1 binds to the conserved cAMP response element (CRE) on the promoter to promote gene transcription and activate phosphorylation[49, 50]. The literature report that CREB1 could suppresses CRC proliferation, invasion and metastasis [51]. Li showed that knockdown of CREB1 exerted effects on proliferation, migration and invasion of CRC cells[52]. RHOA, as the member of a ras homolog gene family, member might affect the domain of E-cadherin and endocytosis[53]. Epidermal growth factor receptor(EGFR) was associated with the the FOXK2 and mediated CRC metastasis. Besides, CRC metastasis was inhibited significantly via the EGFR monoclonal antibody cetuximab[54].

In the present study, we investigated the potential miRNA–mRNA regulatory network in CRLM. But there are still shortcomings. Firstly, we targeted to miRNAs–mRNAs network between CRC and CRLM, however, some of these underlying mechanism of CRLM need to be further confirmed. Secondly, compared with the number of the sample usually required for biomarker analysis, the sample of the article used here was not enough. Thirdly, the miRNA–mRNA only associated with the public databases, and the lack of the experiments confirmed in vivo and in vitro to validate our analysis.

Conclusion

In summary, through the GEO database and bioinformatics analysis, we constructed potential miRNA–mRNA network(hsa-miR-95-3p-CREB1, hsa-mir-122-5p-RHOA and EGFR) in CRLM. We hope that these findings will contribute to further research to improve the prognosis of patients with CRLM.

Data Availability

All data was obtained from the public database described in Materials and Methods and carried out in accordance with relevant guidelines and regulations.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Si-ming Zhang, Cheng Shen, Xiao-hui Jiang and Junlan Qiu participated in the study design and data collection. Si-qiu Zhai, Ling-ling Shi, Jing Li and Xing-dan Wang analyzed the data. Si-ming Zhang and Cheng Shen drafted the manuscript. All the authors read and approved the submitted version of the manuscript. Siming Zhang and Cheng Shen contributed equally to this work.

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The results obtained in this study are partially based on data produced by the open source

of GEO dataset, so there are no ethical issues and other conflicts of interest.

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