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Identification of critical TF-miRNA-mRNA regulation loops for colorectal cancer metastasis

C. Wang[1,2], D.Z. Hu[1,2] and J.Z. Liu[1,2]

[1]Tianjin Medical University Cancer Institute and Hospital,
National Clinical Research Center for Cancer, Tianjin, China
[2]Key Laboratory of Cancer Prevention and Therapy, Tianjin, China

Corresponding author: J.Z. Liu
E-mail: Do_JZLiu@126.com

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ABSTRACT. To explore the potential cause of colorectal cancer metastasis, gene expression profiles, GSE21510, and miRNA expression profiles, GSE48074, were downloaded from the Gene Expression Omnibus database. Differentially expressed genes in metastatic colorectal and non metastatic colorectal cancer compared with the normal samples were identified via the limma package in R. The differentially expressed miRNAs in colorectal cancer samples with lymph node metastasis compared with those without lymph node metastasis were screened out by the some method. Differentially expressed genes that were upregulated in colorectal cancer samples with distant metastasis in comparison to that in samples without distant metastasis and normal samples were considered to play important roles in colorectal cancer metastasis. Functional enrichment analysis of these genes was conducted using the Database for Annotation, Visualization, and Integrated Discovery v6.7. Biological processes related to cell differentiation and cell proliferation were significantly enriched. TF (transcription factor)-miRNA-mRNA regulation loops were constructed by using the starBase and ChIPBase databases. Finally, six critical regulation loops were screened out. They were composed of two

TFs, two miRNAs, and three mRNAs. Some of these TFs, mRNAs, or miRNAs have previously been identified as critical targets in colorectal cancer metastasis. Additionally, several new targets were identified in our study, which may be helpful to improve metastatic colorectal cancer treatment.

Key words: ChIPBase; Colorectal cancer; DAVID; TF-miRNA-mRNA; Gene Expression Omnibus; StarBase

INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies with high morbidity and mortality (Coghlin and Murray, 2015). In some developed Chinese cities such as Shanghai and Hong Kong, it has become the second most common cancer (Li and Ma, 2014). Metastasis is the major cause of death related to CRC (Zhou et al., 2014) and the most common distant metastasis site is the liver (Lupinacci et al., 2014). Some treatments for CRC with or without metastasis have been developed and oxaliplatin-based regimens are the most commonly used treatments to improve survival (Morris-Stiff et al., 2014). However, tumor metastasis is a complex process and its mechanisms remain ambiguous. Herszenyi et al. (2014) proved that proteolytic enzymes play an important role in CRC metastasis. Yesudhas et al. (2014) reported that *TLR-4* was a critical target in CRC metastasis. In this study, we focused on exploring the potential cause of CRC metastasis and on screening molecular targets by the analysis of gene and miRNA expression profiles from microarray data.

miRNA is a small non-coding RNA with a length of 20-25 nucleotides. It is recognized as an important factor in gene regulation (Joshi et al., 2014; Zhu et al., 2014). miRNAs can target a gene and induce the repression of translation or post-transcriptional RNA degradation (Hu and Tang, 2014; Zhang and Wang, 2014). The deregulation of some miRNAs has been proven to be related to some diseases, including cancer. Shiah et al. (2014) proved that *miR-329* and *miR-410* were associated with the pathogenesis of oral squamous carcinoma through the analysis of miRNA expression profiles. A transcription factor (TF) is a protein that controls the transcription process through binding to specific sequences in a gene regulatory region (Latchman, 1993). TF can not only directly regulate the expression of mRNAs, but also that of miRNAs. Thus, TF deregulation may result in the deregulation of miRNAs or mRNAs and induce the appearance of some physical abnormalities.

RNA microarray, a method developed more than 20 years ago, can be used to quantitatively measure gene expression in a very high throughput manner (Debouck and Goodfellow, 1999). It becomes possible to explore the mechanisms or signatures for diseases at the gene level using RNA microarray. Using RNA microarrays, Xu et al. (2013) obtained a 18-gene signature for CRC through the analysis of differentially expressed genes (DEGs) in 216 patients with CRC than that in 181 controls. Clifford et al. (2010) found two single nucleotide polymorphisms that were significantly different between liver cancer samples and normal samples. The large amount of expression profiles generated from RNA microarray are stored in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), in which the data can be freely interpreted (Barrett et al., 2011).

The starBase (http://starbase.sysu.edu.cn/) is a database that deciphers the regulation relationships between RNA and RNA, or protein and RNA by managing the 108 CLIP-Seq datasets from 37 independent studies (Li et al., 2014). It can provide miRNA-mRNA regulation pairs when using a different parameter setting such as the number of CLIP-Seq experiments that can support the pair and the number of software that predict the pair. The ChIPBase (http://deepbase.sysu.edu.cn/chipbase/) is an integrated resource and platform to decode TF binding sites, expression profiles, and transcriptional regulation of non-coding RNAs such as lncRNA, miRNA, and lincRNA via the interpretation of data from 543 CHIP-Seq experiments (Yang et al., 2013). The ChIPBase can provide TF-miRNA regulation pairs when using different parameter settings such as the regulatory regions.

In this study, through the analysis of CRC mRNA and miRNA expression profiles from the GEO database, we investigated the potential cause of CRC metastasis. As a result, six TF-miRNA-mRNA regulation loops that may play critical roles were identified. The TFs, miRNAs, and mRNAs in those loops may serve as important targets for the treatment of metastatic CRC. The workflow of this study is presented in Figure 1.

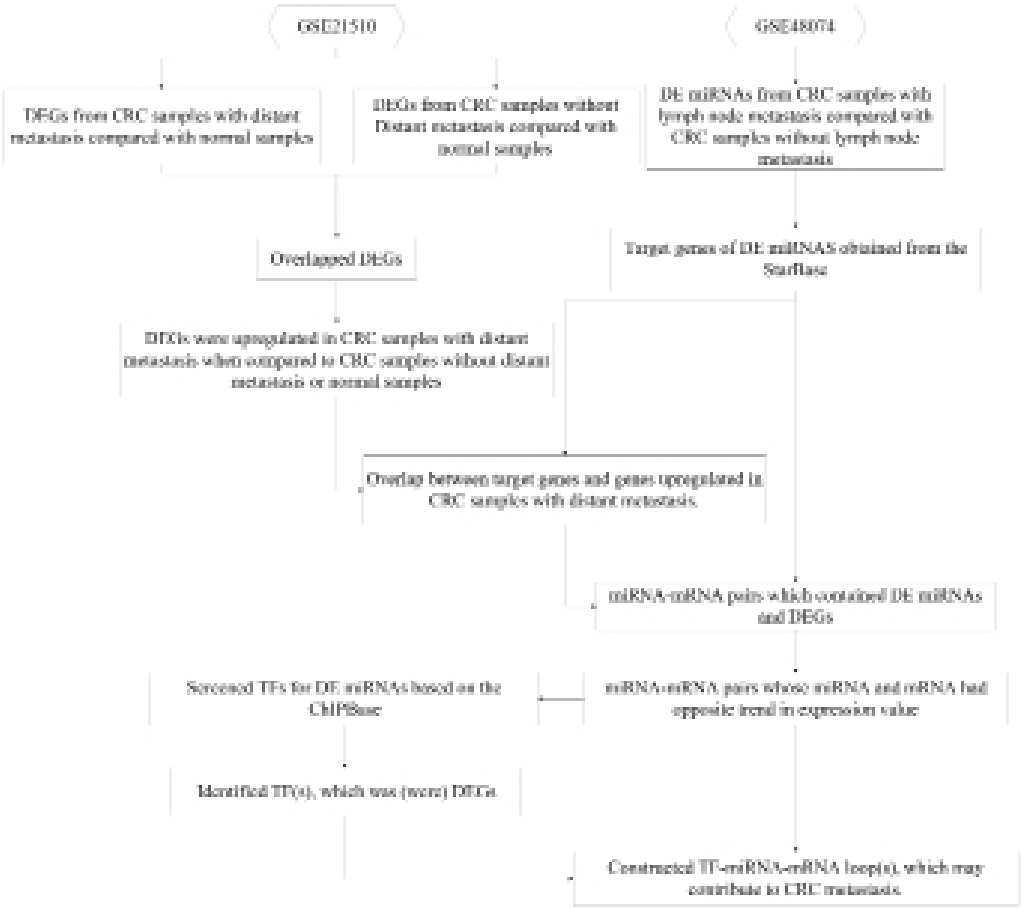

Figure 1. Study workflow.

MATERIAL AND METHODS

Microarray data

The mRNA expression profiles were downloaded from the GEO database using the accession No. GSE21510 (Tsukamoto et al., 2011), which contains a total of 148 samples, including 18 normal colorectal tissue samples, 76 CRC samples without distant metastasis, and 54 CRC samples with distant metastasis. The GSE21510 dataset is based on GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. The miRNA expression profiles (GSE48074) based on GPL11487 Aglient-021827 Human miRNA Microarray [miRNA_107_Sep09_2_105] were also downloaded from the GEO database. Briefly, it is composed of four CRC samples with lymph node metastasis and four CRC samples without lymph node metastasis.

Preprocessing of the microarray data

The raw data in CEL format were downloaded and preprocessed by using R (Huber et al., 2015). Briefly, the expression values were log2-tansformed and standardized by using the robust multi-array average (RMA) method. The probe IDs were then changed into gene symbols by using annotation packages for the mRNA and miRNA microarray platforms. Finally, we summarized the expression levels of the probe sets according to gene symbols.

Identification of DEGs/differentially expressed (DE) miRNAs

The limma (Diboun et al., 2006) package in R was used to identify DEGs and DE miRNAs. Genes and miRNAs with $P < 0.05$ and $|\log2(\text{fold change})| > 1$ were kept. Here, two groups of DEGs (DEGs from CRC samples with/without distant metastasis than those of normal samples) and one group of DE miRNAs (DE miRNAs from CRC samples with lymph node metastasis than those of CRC samples without lymph node metastasis) were screened out.

Identification of critical DEGs for CRC metastasis

Gaiteri et al. (2014) indicated that, if the average expression level of a gene module was higher in patient samples than in normal samples, the genes in this module are more likely to be related to the cause of the disease. To identify the critical DEGs involved in CRC metastasis, we detected DEGs that were upregulated in CRC samples with distant metastasis than those of CRC samples without distant metastasis and normal samples. These genes were referred to as DEG1. Functional enrichment analysis of DEG1 was conducted by using the Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/) v6.7 and GO terms with $P < 0.05$ were screened out.

Identification of critical TF-miRNA-mRNA regulation loops

We obtained the miRNA-mRNA regulation pairs for the DE miRNAs based on the following parameter settings: Number of supported experiments ≥ 1 (at least one CLIP-Seq

experiment supported the miRNA-mRNA pair), number of cancer types ≥ 1 (miRNA expression and its target mRNA were inversely correlated in at least one cancer type), and program number ≥ 1 (at least one software predicted the miRNA-mRNA pair) in the starBase. We retained the miRNA-mRNA pairs if the mRNA belonged to the DEG1 group and miRNA expression level was inversely correlated to the target mRNA expression level in this study. For miRNAs in miRNA-mRNA pairs, we obtained the TF-miRNA regulation pairs based on the ChIPBase setting with upstream and downstream to 5-kb regulatory regions. TF-miRNA pairs in which the TF belongs to the DEGs of CRC samples with or without distant metastasis and for which TF and miRNA expression levels were positively correlated were screened out. The critical TF-miRNA-mRNA regulation loops were obtained based on these TF-miRNA and miRNA-mRNA pairs.

RESULTS

DEGs and DE miRNAs

With a cutoff of $P < 0.05$ and $|\log2(\text{fold change})| > 1$, a total of 2851 DEGs from CRC samples without distant metastasis and 2029 DEGs from CRC samples with distant metastasis were identified when compared with normal samples. There were 1994 overlapped DEGs among the two DEG datasets. Moreover, 7 DE miRNAs from CRC samples with lymph node metastasis were identified than those of CRC samples without lymph node metastasis. These include 5 downregulated and 2 upregulated miRNAs (Table 1).

Table 1. Comparison of differentially expressed miRNAs in CRC samples with lymph node metastasis and CRC samples without lymph node metastasis.

miRNA	Log FC	P value
hsa-miR-152	1.248181	0.001254
hsa-miR-551b	1.431937	0.001514
hsa-miR-19b	-1.1882	0.020905
hsa-miR-29b	-1.32928	0.031561
hsa-miR-99a	1.202455	0.031916
hsa-miR-141	-1.42915	0.044461
hsa-miR-194	-1.01124	0.047281

Log FC: log2 (fold change).

Critical DEGs and functional enrichment analysis

Among the 1994 overlapping genes, 49 genes were found to be upregulated in CRC samples with distant metastasis than in the ones without distant metastasis and normal samples (Table 2). The heatmap of the average expression level of these 49 genes in CRC samples with/without distant metastasis and normal samples are shown in Figure 2. Functional enrichment analysis indicated that these genes are mainly involved in the process of cell differentiation, cell proliferation, cell localization, and cell-cell adhesion. Erythrocyte differentiation and homeostasis were the top two most significant GO terms, which have been reported to be involved in CRC metastasis. Ochiai et al. (2014) indicated that erythropoiesis could increase serum iron, which is a convenient predictor of the response to chemotherapy in patients with advanced CRC. Vali et al. (2008) demonstrated that the ATP level in erythrocytes was associated with colorectal liver metastasis. The enriched GO terms are shown in Table 3.

Table 2. Comparison of upregulated DEGs in CRC samples with distant metastasis and CRC samples without distant metastasis or normal samples.

Gene	Log FC1	Log FC2	Gene	Log FC1	Log FC2
AMIGO2	2.48	2.26	LGR6	1.253915	1.068486
BMP4	1.90	1.50	LINC00888	1.202353	1.171287
CLDN2	2.33	2.09	LPL	2.063578	1.299128
CYP4F3	1.15	1.11	LY6E	1.238197	1.176091
DACT1	1.57	1.08	NMU	1.622998	1.573041
DEFA5	1.73	1.65	OR51E1	1.655626	1.582115
DEFA6	1.43	1.34	PHLDA1	1.632322	1.610682
DPYSL2	1.19	1.09	PLAG1	1.619089	1.572133
DSG3	1.29	1.22	PLEKHG4	1.114769	1.023301
EDAR	1.55	1.31	PRTFDC1	1.163461	1.100993
EMG1	1.25	1.24	PTPN13	1.058221	1.000699
ETV4	1.14	1.02	REG1A	1.771995	1.131865
FAN1	1.15	1.13	REG1B	1.730113	1.162919
FAP	2.01	1.94	RNF182	1.365853	1.236283
FLVCR1	1.11	1.01	S100A2	1.464457	1.111458
GEM	1.26	1.14	SKAP1	1.329612	1.101233
GNG4	1.13	1.07	SLC16A4	1.864104	1.796496
GRAMD1A	1.09	1.06	STXBP1	1.497874	1.194649
GSAP	2.07	1.96	TCN1	2.32957	2.002145
GSPT2	1.21	1.17	TIMP1	1.358588	1.304934
HS6ST2	2.44	2.32	TMEM71	1.800485	1.396431
IL33	1.91	1.88	TMTC4	1.110418	1.080725
KLK6	1.56	1.25	TNFRSF11B	1.803088	1.179123
KRT6B	1.50	1.14	TRAF5	1.054841	1.02911
LEMD1	2.03	1.96			

Log FC1: log2 (fold change) of CRC samples with distant metastasis than normal samples; LogFC2: log2 (fold change) of CRC samples without distant metastasis than normal samples.

Table 3. Enriched GO terms for the 49 critical genes.

Category	Term	P value	Genes
BP	Erythrocyte differentiation	0.00613	BMP4, FLVCR1, TIMP1
BP	Erythrocyte homeostasis	0.00790	BMP4, FLVCR1, TIMP1
BP	Growth	0.01350	BMP4, KLK6, LY6E, FLVCR1
BP	Myeloid cell differentiation	0.02664	BMP4, FLVCR1, TIMP1
BP	Regulation of cellular localization	0.02992	BMP4, STXBP1, EDAR, NMU
BP	Homeostasis of cell number	0.03046	BMP4, FLVCR1, TIMP1
BP	Defense response to fungus	0.03499	DEFA6, DEFA5
BP	Killing cells of another organism	0.03764	DEFA6, DEFA5
BP	Cell-cell adhesion	0.03920	AMIGO2, DSG3, STXBP1, CLDN2

Table note: BP: Biological Process.

Critical TF-miRNA-mRNA loops

Based on the starBase, a total of 1977 miRNA-mRNA regulation pairs for the 7 DE miRNAs were obtained, of which four pairs contained mRNAs that belonged to DEG1, (*hsa-miR-29b*)-*PLAG1*, (*hsa-miR-29b*)-*LPL*, (*hsa -miR-141*)-*PLAG1*, and (*hsa-miR-141*)-*STXBP1*. Moreover, miRNA expression levels were negatively correlated with the expression levels of its target mRNA in all four pairs. TFs that may regulate miRNAs identified in the four miRNA-mRNA pairs were obtained from the ChIPBase. Eighty seven TFs were obtained

and three of them belonged to the DEGs from CRC samples with or without distant metastasis, *E2F6*, *Fosl2*, and *TCF7L2*. However, only *Fosl2* and *TCF7L2* expression levels were positively correlated with the expression levels of their target miRNAs and three TF-miRNA regulation pairs were obtained, *Fosl2*-(*hsa-miR-141*), *TCF7L2*-(*hsa-miR-141*), and *TCF7L2*-(*hsa-miR-29b*). Based on the four miRNA-mRNA pairs and the three TF-miRNA pairs, a total of six TF-miRNA-mRNA regulation loops, *Fosl2*-(*hsa-miR-141*)-*PLAG1*, *Fosl2*-(*hsa-miR-141*)-*STXBP1*, *TCF7L2*-(*hsa-miR-141*)-*PLAG1*, *TCF7L2*-(*hsa-miR-141*)-*STXBP1*, *TCF7L2*-(*hsa-miR-29b*)-*PLAG1*, and *TCF7L2*-(*hsa-miR-29b*)-*LPL* were obtained and thought to play important roles in CRC metastasis. The regulation network composed of these regulation loops is shown in Figure 3.

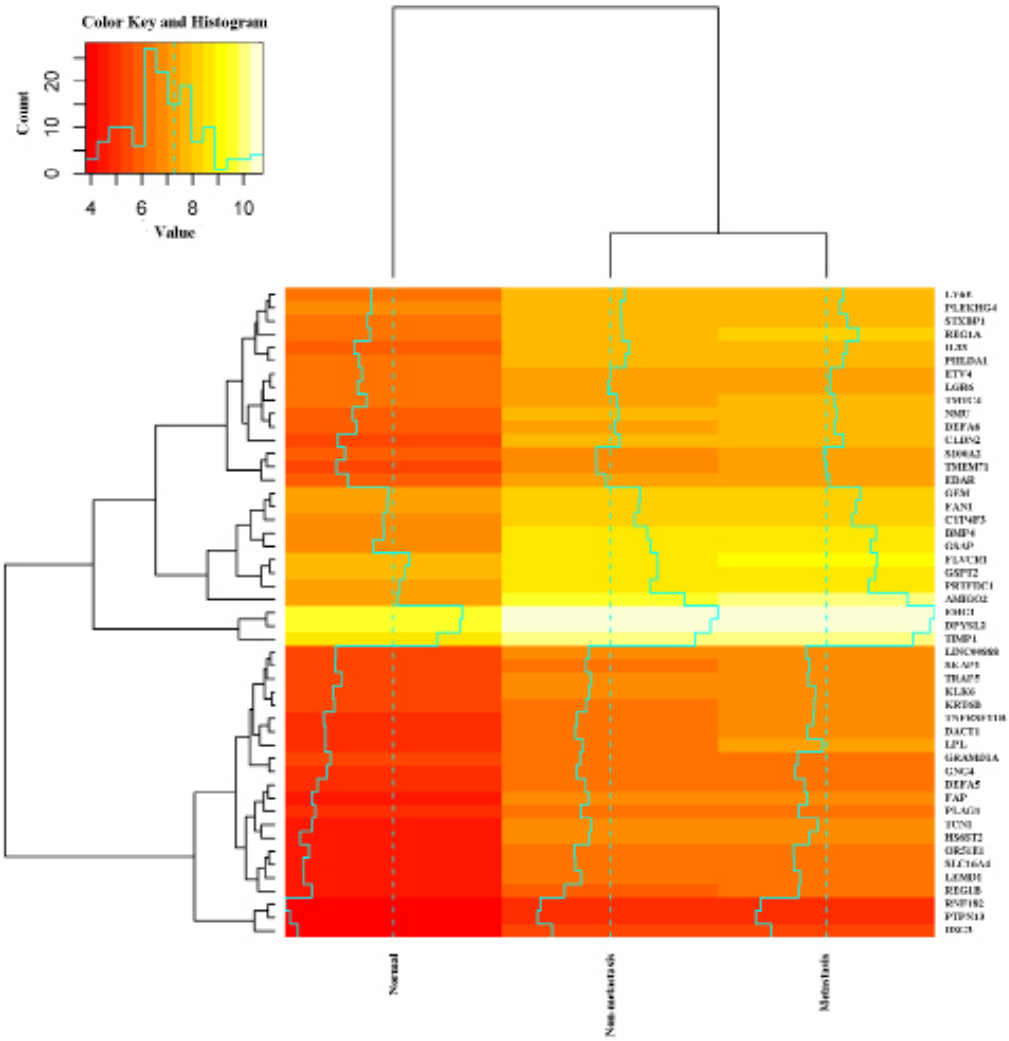

Figure 2. Heatmap of the average expression level of 49 critical genes in CRC samples with/without distant metastasis and normal samples. Metastasis indicates CRC samples with distant metastasis, non-metastasis indicates CRC samples without distant metastasis, and normal indicates normal samples.

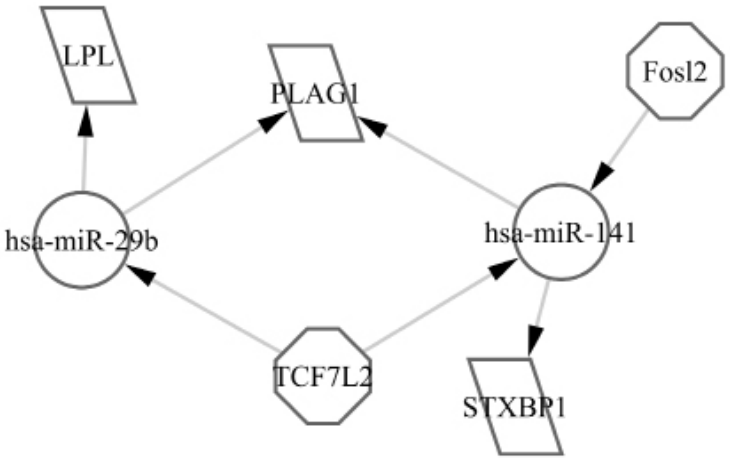

Figure 3. Regulation network composed by the six TF-miRNA-mRNA regulation loops. The octagon nodes represent TFs, circular nodes represent miRNAs, parallelogram nodes represent mRNAs, and arrows represent regulatory relationships.

DISCUSSION

Cancer metastasis is a complex process and many studies have been designed to explore cancer metastasis mechanisms and signatures. In this study, through the analysis of mRNA and miRNA expression profiles from the GEO database, DEGs and DE miRNAs that may be related to CRC metastasis were identified. Functional enrichment analysis of DEGs that are upregulated in CRC samples with distant metastasis than those in CRC samples without distant metastasis and normal samples indicated that these genes are mainly involved in cell differentiation or proliferation. Six critical TF-miRNA-mRNA regulation loops that may play important roles in CRC metastasis were identified.

A total of 49 DEGs were found to be upregulated in CRC samples with distant metastasis than that in CRC samples without distant metastasis and normal samples, these genes are thought to play important roles in CRC metastasis. Biological processes, related to cell proliferation and differentiation, were significantly enriched, which is consistent with previous studies (Arndt et al., 2009, Karagiannis et al., 2013). *BMP4* was found in most of the GO terms. Many studies have clarified the relationship between *BMP4* and CRC initiation or development. In an association study, Yang et al. (2014) found that rs4444235, a cis-acting regulator of *BMP4*, could contribute to CRC risk in Taiwanese patients. The relationship between rs4444235 and CRC was also demonstrated by Lubbe et al. (2012). However, the association between *BMP4* and CRC metastasis remains unclear and further studies are still needed.

Based on the starBase and ChIPBase databases, six TF-miRNA-mRNA regulation loops that may play important roles in CRC metastasis were identified. They include two TFs, two miRNAs, and three mRNAs. In the regulation loops, *TCF7L2* regulates both miRNAs. As a transcript factor, *TCF7L2* plays a key role in the Wnt signaling pathway, which has been demonstrated to be involved in CRC (Ashktorab et al., 2014, Hu et al., 2014, Planutis et al., 2014). Additionally, the relationship between *TF7L2* and CRC metastasis has been revealed

in previous studies. Through whole genome sequencing, Shanmugam et al. (2014) detected a *TCF7L2* mutation in metastatic CRC. In a large scale genetic study, Zhang et al. (2014) found that some loci in *TCF7L2* and *TGFB1* were associated with colorectal tumorigenesis.

hsa-miR-141 regulates two of the three mRNAs in the regulation loops. As a short non-coding RNA, *hsa-miR-141* is involved in the post-transcriptional regulation of many target genes and its deregulation is associated with many types of cancer such as breast (Gregory et al., 2008) and kidney cancer (Nakada et al., 2008) as well as CRC and CRC metastasis. Through the investigation of some miRNA expression profiles, Yin et al. (2014) found that *hsa-miR-141* was a novel biomarker in CRC liver metastasis. Cheng et al. (2011) identified *hsa-miR-141* as a critical biomarker for metastatic CRC that predicts poor prognosis.

PLAG1 was regulated by both miRNAs, although no previous study proved the relationship between *PLAG1* and CRC. However, it has been reported to be involved in other cancers. Dotlic et al. (2014) found that *PLAG1* was involved in the development of neoplasm by using fluorescence *in situ* hybridization, reverse transcription-polymerase chain reaction, and gene microarray. Wang et al. (2013) illustrated the role of *PLAG1* in pleomorphic adenoma through microarray analysis. Thus, *PLAG1* may be a novel signature involved in CRC. In conclusion, through bioinformatics methods, critical targets that may be involved in CRC metastasis were identified. Some of these targets have been previously identified, while others are newly found targets, Wet laboratory experiments are warranted to confirm these newly found targets.

CONCLUSIONS

Overall, bioinformatics analysis of mRNA and miRNA expression profiles identified some TF-miRNA-mRNA regulation loops that may play important roles in CRC metastasis. These results will be helpful in experimental studies and clinical treatment of CRC.

Conflicts of interest

The authors declare no conflict of interest.

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