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Identification of Key Genes in Colorectal Cancer Regulated by miR-34a

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Statistical Analysis C
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Background: The aim of this study was to screen the molecular targets of *miR-34a* in colorectal cancer (CRC) and construct the regulatory network, to gain more insights to the pathogenesis of CRC.

Material/Methods: The microarray data of CRC samples and normal samples (GSE4988), as well as CRC samples transformed with *miR-34a* and non-transfected CRC samples (GSE7754), were downloaded from the Gene Expression Omnibus (GEO) database. The differently expressed genes (DEGs) were identified via the LIMMA package in R language. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to identify significant Gene Ontology (GO) terms and pathways in DEGs. The targets of *miR-34a* were obtained via the miRWalk database, and then the overlaps between them were selected out to construct the regulatory network of *miR-34a* in CRC using the Cytoscape software.

Results: A total of 392 DEGs were identified in CRC samples compared with normal samples, including 239 upregulated genes and 153 downregulated ones. These DEGs were enriched in 75 GO terms and one Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. At the same time, 332 DEGs (188 upregulated and 144 downregulated) were screened out between *miR-34a* transformed CRC and *miR-34a* non-transfected CRC samples and they were enriched in 20 GO terms and eight KEGG pathways. Six overlapped genes were identified in two DEGs groups. There were 1,668 targets of *miR-34a* obtained via the miRWalk database, among which 21 were identified differently expressed in *miR-34a* transformed CRC samples compared with *miR-34a* non-transfected CRC samples. Two regulatory networks of *miR-34a* in CRC within these two groups of overlapped genes were constructed respectively.

Conclusions: Pathways related to cell cycle, DNA replication, oocyte meiosis, and pyrimidine metabolism might play critical roles in the progression of CRC. Several genes such as *SERPINE1, KLF4, SEMA4B, PPARG, CDC45*, and *KIAA0101* might be the targets of *miR-34a* and the potential therapeutic targets of CRC.

MeSH Keywords: Colorectal Neoplasms • Microarray Analysis • MicroRNAs

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Background

Colorectal cancer (CRC) is the third most common cancer and the fourth-leading cause of cancer related death worldwide with an incidence of more than 1,000,000 per year [1,2]. According to statistics, the probability of developing CRC increases from 0.07% in the first four decades of life to 4.5–5% in the seventh decade of life [3,4]. Furthermore, studies suggest that 40–50% of patients who underwent potentially curative surgery ultimately relapsed and died of metastatic disease [5,6]. The most important prognostic indicator of survival in early CRC is the stage of the tumor determined by the depth of penetration through the bowel wall, and the number of involved lymph nodes [6]. However, the tumors are often diagnosed at an intermediate or late stage with poor prognosis, because of the complexity in medical diagnosis. In addition, pathological staging fails to predict recurrence accurately in many patients undergoing surgery for CRC. In fact, 10–20% patients with stage II CRC, and 30–40% of those with stage III CRC, develop recurrence [7]. It is crucial to explore the molecular mechanism and identify reliable biomarkers that can guide the diagnosis and therapy of CRC.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs to regulate gene expression [8], and they were reported to be closely associated with cell growth, proliferation, differentiation, and death [9,10]. There has been increasing evidence indicating that *microRNAs* play important roles in the development of cancers [11], such as the proliferation, apoptosis, invasion, and metastasis of tumors [12,13]. *MicroRNA-34a (miR-34a*) is a pivotal member of the p53 network, and it was found to be downregulated in multiple types of tumors, including CRC [14], and often act as a tumor suppressor [15–19]. Studies have shown that *miR-34a* regulated multiple developmental cell-fate mechanisms, including the differentiation of human embryonic stem cells and somatic cell reprogramming [16,20,21]. Furthermore, was it has also been suggested that *miR-34a* plays critical roles in inhibiting tumor recurrence [22]. However, the specific regulatory mechanism of *miR-34a* in CRC is still unclear.

In this study, we aimed to explore some key targets associated with the development of CRC. And our study might contribute to promoting available biomarkers for the early diagnosis, therapy, and prognosis of CRC.

Material and Methods

Microarray data

Microarray data set GSE4988 and GSE7754 were downloaded from the GEO (*http://www.ncbi.nlm.nih.gov/geo/*) database. The GSE4988 data set contained 20 samples: 12 CRC samples and eight normal samples. These microarray data were analyzed using the GPL3014 (ResGenHs15K_DIO) platform. The GSE7754 dataset contained four samples: two CRC samples transformed with *miR-34a* and two non-transfected CRC samples, and the data were analyzed based on GPL70 (HG-U133_Plus_2, Affymetrix Human Genome U133 Plus 2.0 Array) platform.

Data preprocessing and identification of DEGs

The raw data was background corrected, log2 transformed, and quantile normalized using Robust Multi-array Average (RMA). If multiple probes corresponded to one gene, the mean expression value was defined as expression value. LIMMA package in R language was used to analyze the DEGs in CRC samples compared to normal samples (regarded as DEGs-1), as well as CRC samples transformed with *miR-34a* compared to non-transfected CRC samples (regarded as DEGs-2). DEGs were obtained according to the criteria: adjusted $p<0.05$ and $|\log(\text{fold change})| > 1$.

Functional enrichment analysis of DEGs

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were commonly used approaches for functional and pathway studies of large-scale genomic or transcription data, respectively [23]. GO terms included biological processes (BP), molecular function (MF), and cellular component (CC). The Database for Annotation, Visualization and Integrated Discovery (DAVID) [24] (*https://david.ncifcrf.gov/*) was a widely used web-based tool for the functional annotation of DEGs. To investigate the bio-functions of DEGs, GO, and KEGG pathway analyses were conducted based on the online software DAVID with $p<0.05$.

Construction of the transcriptional regulatory network of miR-34a

MiRWalk (*http://mirwalk.uni-hd.de/*) is a publicly available comprehensive resource, hosting the predicted as well as the experimentally validated microRNA (miRNA)-target interaction pairs. The targets of *miR-34a* were identified based on the miRWalk database, and the overlapped genes between these targets and DEGs-2 were selected. Then the transcriptional regulatory network with *miR-34a* was constructed. At the same time, the overlapped genes between DEGs-1 and DEGs-2 were also selected to build the regulatory network with *miR-34a*.

Verification of related upregulated and downregulated genes

The expression levels of some DEGs were detected in colon cancer cell line HCT116 (observed by our laboratory) by RT-PCR.

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The culture of HCT116 and the retroviral expression of *miR-34a* referred to the methods of Chang et al. [25]. HCT116 cells transformed with *miR-34a* were named as *miR-34a* group, and non-transformed cells as the control group. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 1 μg of total RNA from each sample was subjected to reverse transcription by SuperScript II reverse transcriptase (Invitrogen, America). Reverse transcription was performed at 42°C for one hour, followed by 95°C for five minutes. Quantitative real-time PCR (Q-PCR) was performed to determine the mRNA levels of *SERPINE1, KLF4, SEMA4B, PPARG, CDC45*, and *KIAA0101* using a SYBR® Premix Ex Taq™ Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions and an ABI Prism 7000 Sequence Detection System (Applied Biosystems, CA, USA). The amplification conditions were as follows: 95°C for five minutes; followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 35 seconds; and a final five minute 72°C extension. All the primers were designed and synthesized by Takara Biomedical Technology (Beijing) Co., Ltd. and they are shown in Table 1.

Table 1. Target genes and corresponding primer sets.

Target genes	Primer sets
SERPINE1	Forward: TCTTCGATTGTCACCACCGA
	Reverse: TCAAGCTGGGAGTACTGTGG
KLF4	Forward: CCACCGGACCTACTTACTCG
	Reverse: AAGCCAAAACCCAAAACCCC
SEMA4B	Forward: CACTTGACCCTGTTTCCCAC
	Reverse: CAGCTCCTCAGGCCCATC
PPARG	Forward: AAGGAGTCAGAAACGGGGAG
	Reverse: TGGCATCTCTGTGTCAACCA
CDC45	Forward: TAGGCCAGTCAATGTCGTCA
	Reverse: GAAGGCTCTGACCCATCACT
KIAA0101	Forward: CAGAAAAGGTGAGGCGATCG
	Reverse: ATAACCTTACCCTGCCCCTG

Statistical analysis

SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analyses, and data were expressed as the mean ±SEM. The *t*-test was used to compare both two groups, and *p* values <0.05 were considered statistically significant.

Results

Identification of DEGs

After data preprocessing, a total of 392 DEGs were identified in DEGs-1. Among these genes, 239 were upregulated and 153 were downregulated (Figure 1A). At the same time, the DEGs-2 set contained 332 DEGs (188 upregulated and 144 downregulated), and the top 20 DEGs of are shown in Figure 1B. The hierarchical clustering of DEGs in CRC samples, normal samples, *miR-34a* transformed CRC samples, and non-transfected CRC samples are shown in Figure 1C and 1D, respectively. The top 20 DEGs of DEGs-1 and DEGs-2 were separately listed in Table 2A and Table 2B.

Function enrichment of DEGs

There were 75 enriched GO terms of DEGs-1 identified, including 49 biological processes (BP), 19 cellular component (CC) and seven molecular function (MF) terms. Then, one KEGG pathway related to basal transcription factors was obtained. In DEGs-2, 243 GO terms and eight KEGG pathways were obtained. The top 10 GO terms for DEGs-1 and DEGs-2 are shown in Figure 2. Table 3 shows the enriched KEGG pathways of DEGs-1 and DEGs-2.

Analysis of regulatory network of miR-34a in CRC

There were 1,668 target genes of *miR-34a* obtained via the miRWalk database, and 21 overlaps with DEGs-2 were identified, among which 11 genes (*NTN4, KLF4, CEBPG, SEMA4B, PPARG, SERPINE1, C11orf38, FGD6, THBD, RTN1,* and *RRAGD*) were downregulated. At the same time, six overlapped genes (*KNTC1, CDC45, HAT1, DLGAP5, KIAA0101,* and *FAM64A*) were identified in the DEGs-1 and DEGs-2. Figure 3 shows the regulatory network between *miR-34a* and these overlapped genes.

Verification of important genes

Several genes such as *SERPINE1* (downregulated), *KLF4* (downregulated), *SEMA4B* (downregulated), *PPARG* (downregulated), *CDC45* (upregulated) and *KIAA0101* (upregulated) might be the targets of *miR-34a*. Results of RT-PCR are shown in Figure 4, which verified the related upregulated and downregulation of the aforementioned DEGs in CRC cells transformed with *miR-34a* compared with those non-transfected cells.

Discussion

Despite advances in technologies of detection and therapies, CRC is still an uncontrollable disease. The lifetime risk of developing CRC is about 5.1% [26]. In the United States, CRC accounts for approximately 10% of cancer cases and cancer-related deaths [4]. *MiR-34a* was found to be a tumor suppressor

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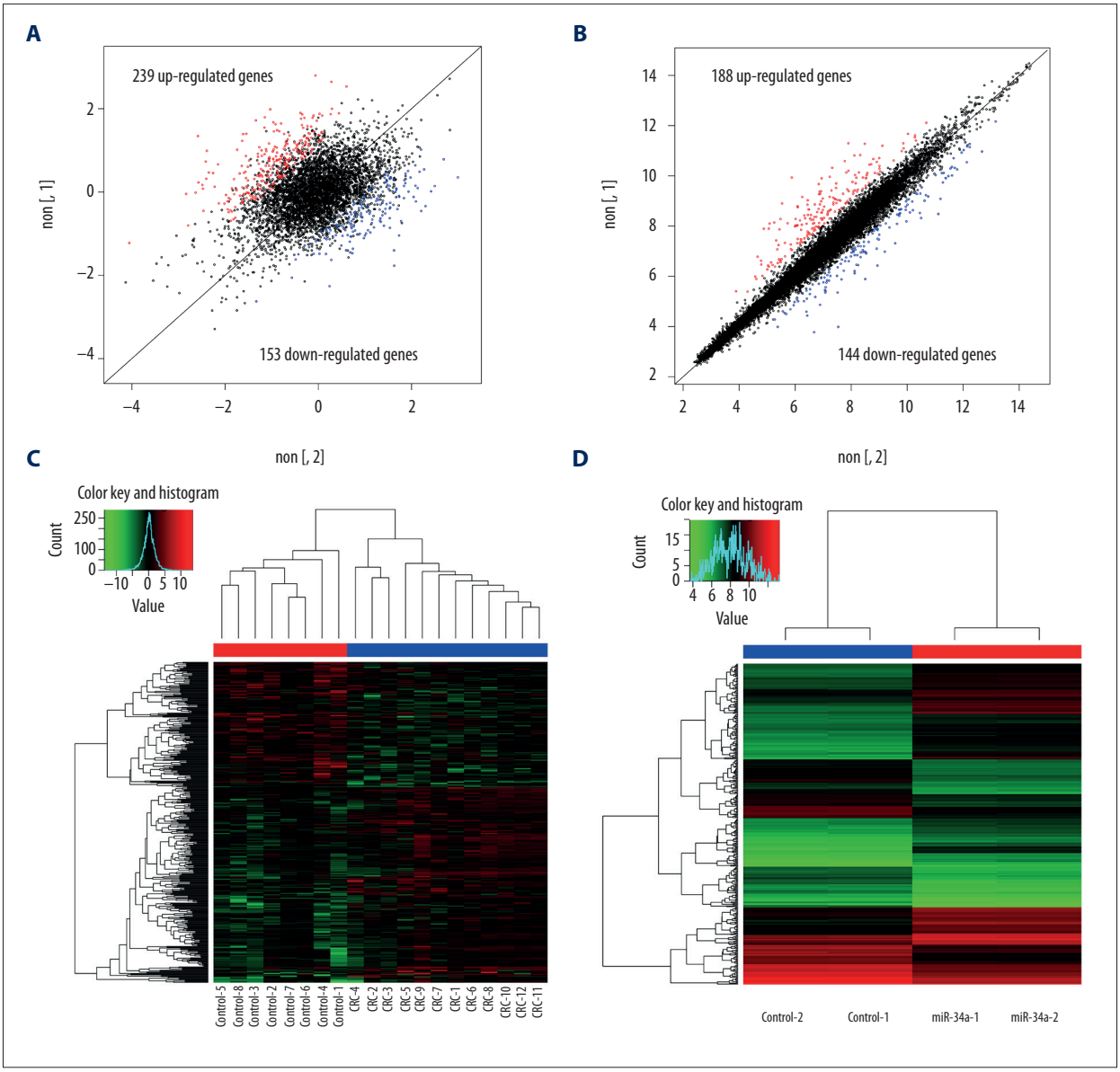

Figure 1. The DEGs change trend (**A, B**) and hierarchical cluster analysis of samples (**C, D**) in the data set of GSE4988 (**A, C**) and GSE7754 (**B, D**). Colors in the heat map represent for different gene expression level. Based on DEGs-1, CRC samples and normal samples were classified to different clusters (**C**). Similarly, *miR-34a* transformed CRC samples and non-transfected CRC samples were classified to different clusters according to DEGs-2 (**D**).

in various cancers. In our study, DEGs, GO terms, and KEGG pathways in CRC samples and *miR-34a* transformed CRC samples were identified. Through the construction of the regulatory network of *miR-34a*, key genes in the progression of CRC were obtained, which might help make better understanding of the molecular mechanism and provide important reference for the diagnosis and therapy of CRC.

GO functional enrichment analysis indicated that both DEGs-1 and DEGs-2 were enriched in cell differentiation and cell cycle related biological processes. These processes were reported to be closely related to cancers. The majority of human cells were not cycling, while the minority of cells were cycling and were mainly located in self-renewing tissues. Deregulation of the cell cycle under laid the aberrant cell proliferation that characterized cancer [27], and tumor cells could not receive sufficient mitogenic signaling to drive them through the cycle and the division. At the same time, cell cycle phases could also be prognostic markers and therapy targets in various of cancers [28,29]. DEGs-2 has also been shown to be related to the biosynthesis of proteins and some other substances. Studies have shown that the rate of protein synthesis was a key factor

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Table 2A. The top 20 DEGs of CRC samples compared to normal samples.

Gene name	*P* value	LogFC
DD×46	4.49×10^{-05}	2.04625
ARMC1	0.000199	3.348333
HNRNPH3	0.000284	1.926875
FAM46A	0.000446	–2.04625
EPAS1	0.000526	2.98625
HNRNPLL	0.000576	2.830833
CRAT	0.000714	3.89375
TNKS	0.000876	–1.9825
GPR98	0.0009	–2.80917
CCNT1	0.00093	–2.43167
CB×1	0.001027	1.990417
DGKZ	0.001154	1.722083
CD83	0.001174	2.910417
BAD	0.001219	–1.9975
IPO11	0.001407	1.87375
TRIP10	0.001578	–1.94583
SMN2	0.001641	1.835
RNF139	0.001666	2.78625
EIF3A	0.001693	3.027083
MCL1	0.001921	2.909583

DEGs – differentially expressed genes; FC – fold change; CRC – colorectal cancer.

Table 2B. The top 20 DEGs of *miR-34a* transformed CRC samples compared to *miR-34a* non-transfected CRC samples.

Gene name	P value	LogFC
RRM2	2.82×10^{-09}	3.34178
NCAPG	6.47×10^{-09}	2.849829
PBK	7.61×10^{-09}	2.939017
ANLN	7.74×10^{-09}	2.775134
NMU	9.14×10^{-09}	2.73189
TMEM158	9.14×10^{-09}	–2.68421
CCNB1	9.31×10^{-09}	2.897966
KIF11	9.33×10^{-09}	2.829634
ANKRD29	9.56×10^{-09}	–2.66631
BUB1B	1.17×10^{-08}	2.667052
LURAP1L	1.35×10^{-08}	–2.56479
CDC20	1.50×10^{-08}	2.758017
ZWINT	1.52×10^{-08}	2.640488
MAD2L1	2.08×10^{-08}	2.402782
SHCBP1	2.32×10^{-08}	2.956084
PRC1	2.59×10^{-08}	2.241771
MND1	2.87×10^{-08}	2.368264
KIF14	3.04×10^{-08}	2.958099
STK39	3.52×10^{-08}	–2.22861
MKI67	3.73×10^{-08}	2.522868

DEGs – differentially expressed genes; FC – fold change; CRC – colorectal cancer.

affecting the protein folding in endoplasmic reticulum (ER), and the proteins misfolding in ER involved in the process of various of cancers [30]. Furthermore, protein synthesis was a significant process of metabolism and was closely related to many biological processes, such as cell cycle and cell differentiation. Studies showed that inhibition of mitochondrial protein synthesis lead to the lack of oxidative ATP generating capacity, which resulted in proliferation arrest of normal and malignant cells [31]. KEGG enrichment analysis showed that these DEGs were mainly related to cell cycle related pathway, pyrimidine metabolism related pathway, oocyte meiosis related pathway and DNA replication related pathway. Overall, they were all associated with cell growth, cell invasion, cell proliferation, and cell cycle, all of which play critical roles in the process of tumorigenesis [32]. Research has shown that *miR-34a* suppress tumors and inhibits recurrence of CRC through inhibiting cell growth, migration, and invasion, inducing cell apoptosis and cell cycle arrest [22,33]. Several target genes involved in these processes have been reported to be regulated by *miR-34a* so as to affect the pathogenetic process of CRC, such as *SIRT1* and *NOTCH1* [16,34]. The GO terms and KEGG pathways obtained in our study were in accordance with these other studies and indicated the function of *miR-34a* in regulating the progression of CRC.

There were 21 overlapped genes identified in DEGs-2 and the targets of *miR-34a*, among which 11 genes were downregulated in *miR-34a* transformed CRC samples, including *NTN4, KLF4, CEBPG, SEMA4B, PPARG, SERPINE1, C11orf38, FGD6, THBD, RTN1, and RRAGD*. *MiR-34a* was identified to be a tumor suppressor in many types of solid tumor. It was reported that microRNAs usually act as a silencer of gene expression by binding to the 30 untranslated regions (30 UTRs) of target mRNAs, inhibiting their translation or marking them for degradation [17], so that the downregulated genes might be more reliable targets of *miR-34a* in the progression of CRC. Among

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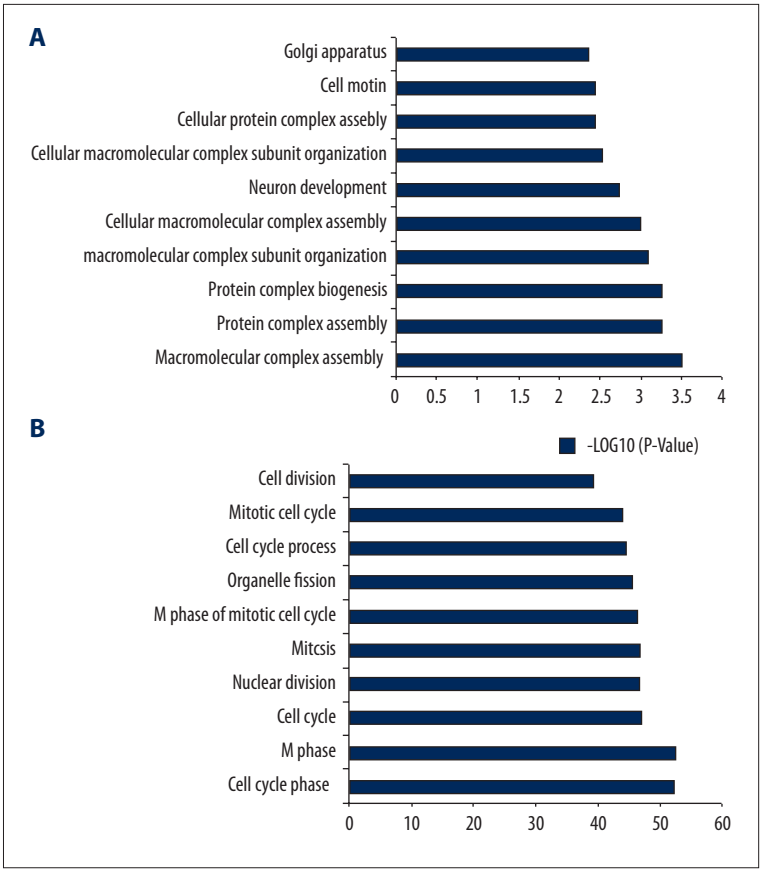

Figure 2. The top 10 GO terms for which the DEGs-1 (**A**) and the DEGs-2 (**B**) were enriched.

Table 3. The enriched KEGG pathway for DEGs of CRC samples compared to normal samples, as well as DEGs of miR-34a transformed CRC samples compared to miR-34a non-transfected CRC samples.

Category	Pathway name	Gene number	P value
KEGG pathway for DEGs-1			
KEGG pathway	Basal transcription factors	11	0.025411
KEGG pathways for DEGs-2			
KEGG pathway	Cell cycle	23	1.39×10^{-14}
KEGG pathway	DNA replication	13	6.43×10^{-12}
KEGG pathway	Oocyte meiosis	12	2.69×10^{-05}
KEGG pathway	Pyrimidine metabolism	10	2.28×10^{-04}
KEGG pathway	Progesterone-mediated oocyte maturation	8	0.002809
KEGG pathway	p53 signaling pathway	7	0.003764
KEGG pathway	pathways in cancer	15	0.013212
KEGG pathway	Base excision repair	4	0.041781

DEGs-1 – differentially expressed genes in colorectal samples compared to normal samples; DEGs-2 – differentially expressed genes in *miR-34a* transformed colorectal samples compared to *miR-34a* non-transfected colorectal samples. KEGG – Kyoto Encyclopedia of Genes and Genomes.

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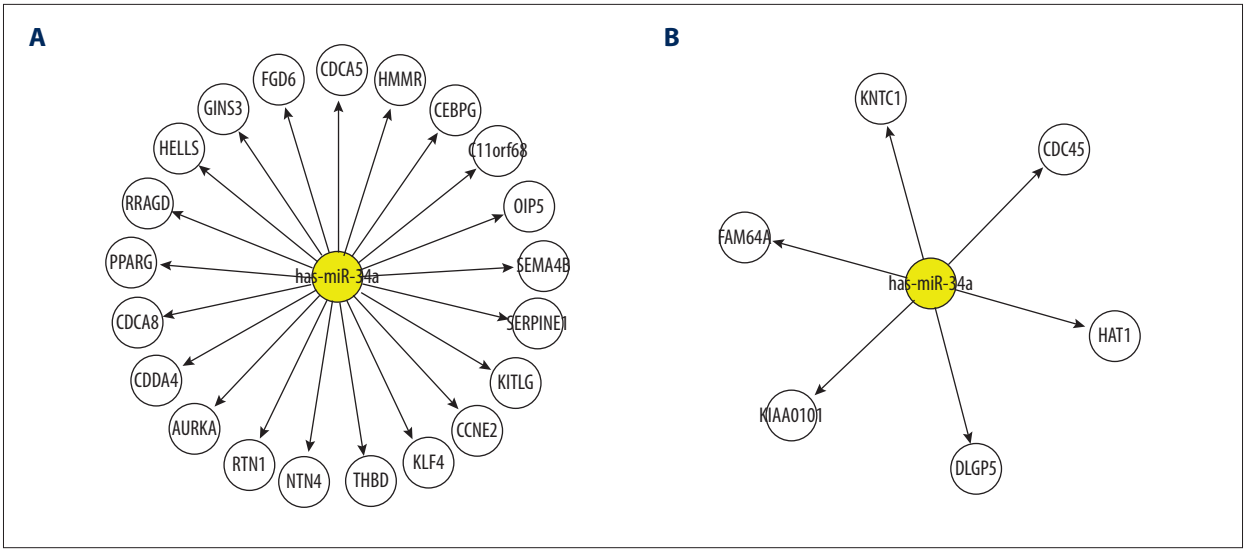

Figure 3. Regulatory network between *miR-34a* and the overlapped DEGs. Genes in A were the overlaps between DEGs-2 and the targets of *miR-34a*, well genes in B were the overlaps between DEGs-1 and DEGs-2.

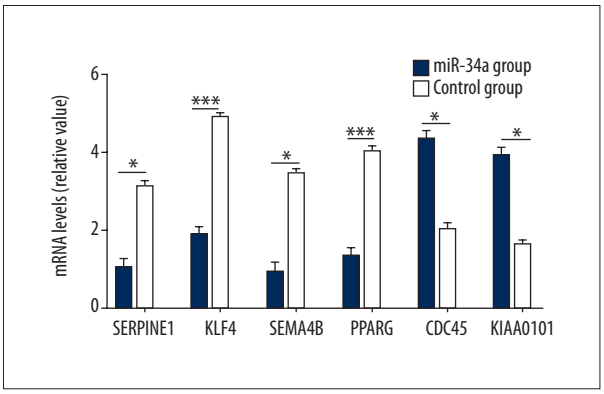

Figure 4. The mRNA expression levels of ERPINE1, KLF4, SEMA4B, PPARG, CDC45 and KIAA0101 in miR-34a group and control group. * meant $p<0.05$, *** meant $p<0.001$.

these genes, *NTN4, KLF4, SERPINE1*, and *SEMA4B* played critical roles in regulating cell growth, migration, and invasion in various of cancers, including CRC [9,35–37]. Studies showed that the expression of *SERPINE1* was increased in CRC and was related to tumor invasiveness and aggressiveness [36]. *KLF4* was an important factor in regulating cell cycle [38], which played important roles in the progression of CRC. Other genes such as *CEBPG* and *PPARG* were also reported to be related to the progression of cancers [39,40]. Many of these genes, such as *KLF4* and *PPARG*, were reported to be regulatory targets of *miR-34a* [41,42]. There were studies that showed that *miR-34a* regulated apoptosis in liver cells by targeting the *KLF4* gene [41]. These results indicated the critical roles of *miR-34a* in CRC, and indicated that *miR-34a* could affect the progression of CRC by regulating the expressions of genes related to cell migration, invasion, and other tumor related functions.

At the same time, six genes (*KNTC1, CDC45, HAT1, DLGAP5, KIAA0101*, and *FAM64A*), were identified to be overlaps between DEGs-1 and DEGs-2, indicating their potential functions in the progression of CRC and their relationship with *miR-34a*. These genes were also reported to be biomarkers or to be closely linked to cell migration, invasion, or DNA replication in various of cancers [43–46]. *KIAA0101* was a p15PAF (proliferating cell nuclear antigen (PCNA)-associated factor) to bind with PCNA. Studies have shown that *KIAA0101* was overexpressed in pancreatic cancer cells, and knocking down of *KIAA0101* by small interfering RNA in pancreatic cancer cells caused drastic attenuation of cell proliferation, well exogenous overexpression of *KIAA0101* enhanced cancer cell growth [45]. *CDC45* plays a critical role in DNA replication. Studies showed that the expression of *CDC45* was tightly associated with proliferating cell populations, and *CDC45* seemed to be a promising candidate for a novel proliferation marker in cancer cell biology [46]. In our study, these overlapped genes were differently expressed in DEGs-1, DEGs-2, and the targets of *miR-34a*; thus we surmised that *miR-34a* might affect the progression of CRC by regulating the expression of these tumor related genes directly or indirectly.

Conclusions

In summary, pathways related to cell cycle, DNA replication, oocyte meiosis, and pyrimidine metabolism might be associated with CRC. *MiR-34a* plays an important role in regulating the progression of CRC and may provide an important reference for the diagnosis and treatment of CRC. Several genes such as *SERPINE1, KLF4, SEMA4B, PPARG, CDC45*, and *KIAA0101* might be the targets of *miR-34a* and the potential therapeutic

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targets of CRC. Our study helps provide a better understanding of *miR-34a* in CRC and may provide important reference for the diagnosis and treatment of CRC. However, further experiments are still needed to confirm the results and to explore the specific regulation mechanism between *miR-34a* and these targets.

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Conflict of interests

None.

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