

Received: 2019.01.16

Accepted: 2019.03.02

Published: 2019.03.18

Analysis of Differentially Expressed lncRNAs and mRNAs for the Identification of Hypoxia-Regulated Angiogenic Genes in Colorectal Cancer by RNA-Seq

Authors' Contribution:

Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

AE 1 **Yi Han***
CE 1 **Xiaoyan Wang***
BF 2 **Enqiang Mao**
DF 1 **Baiyong Shen**
AF 1 **Liang Huang**

1 Department of General Surgery, Ruijin Hospital Affiliated Shanghai Jiaotong University School of Medicine, Shanghai, P.R. China
2 Department of Emergency, Ruijin Hospital Affiliated Shanghai Jiaotong University School of Medicine, Shanghai, P.R. China

* Yi Han and Xiaoyan Wang contributed equally to this work

Corresponding Author: Liang Huang, e-mail: hl6522@126.com

Source of support: Departmental sources

Background: Hypoxia is an important feature of solid tumors and related to a perturbed blood supply in pathophysiology. The aim of our research was to analyze the hypoxia response and elaborate its potential functions in colorectal cancer.





Material/Methods: The lncRNAs and mRNAs expression profile were analyzed in colorectal cancer cell line SW480 by RNA sequencing, and the functions and pathways of differentially expressed genes were screened by Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analysis.

Results: In this study, 77 lncRNAs and 1327 mRNAs were identified as differentially expressed. We discovered several novel lncRNAs, such as RP11-126K1.2, RP3-438O4.4, LINC01119, CTB-22K21.2, RP11-798M19.6, and RP11-2B6.3, which had not been previously reported in regulation by hypoxia. KEGG and GO analyses identified that the differentially expressed changes in mRNAs were mainly related to regulation of basic metabolic processes and gene transcription processes and were involved in several classical pathways which were linked to cancer.

Conclusions: Taken together, the present findings elucidate a set of differentially expressed lncRNAs and mRNAs involved in the hypoxia response process of colorectal cancer, which may serve as a candidate diagnostic biomarker and help to explain the mechanism of initial event in colorectal carcinogenesis in colorectal cancer.

MeSH Keywords: **Cell Hypoxia • Colorectal Neoplasms • RNA, Long Noncoding • Sequence Analysis, RNA**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/915179>

 1594  1  4  19



Background

Colorectal cancer is the third most common malignancy in the world. Mortality has improved significantly over the past few decades due to improved treatment and early diagnosis, but there are still about 1 million new diagnosed people with colorectal cancer each year [1]. A large number of studies have identified smoking, obesity, epigenetic and genetic factors as risk factors for colorectal cancer. However, the exact molecular mechanism of this neoplasm is still unknown. It is still necessary to find new tumor markers and treatment methods to improve the prognosis of patients with colorectal cancer.

Long noncoding RNAs (lncRNAs) are a class of noncoding RNA transcripts that have no protein-coding capacity with larger than 200 nucleotides [2]. Several studies have shown the lncRNAs could mediated gene expression regulation to modulate key cellular processes such as apoptosis, migration, cell proliferation, and autophagy. The length of lncRNAs enables them to fold into intricate structures and work as RNA sequences through secondary and tertiary structural determinants [3]. Moreover, evidence shows that there is a connection between lncRNAs and microRNAs (miRNAs) and lncRNAs have ability to regulate miRNA activity, by functioning as either sponges for miRNAs or competitive endogenous RNAs.

Hypoxia is an important feature of solid tumors and related to a perturbed blood supply in pathophysiologies [4]. It is closely related to tumor angiogenesis, distant metastasis, and chemotherapy or radiotherapy resistance in colorectal cancer [5–7]. Due to a cancer specific characteristic and a key regulatory role in tumor growth, hypoxia has been regarded as one of the best validated cancer selective targets [8]. Colorectal cancer cells in the hypoxic regions could turn on a transcriptional response mainly mediated by the hypoxia-inducible factors (HIFs), then help them survive and grow [5,9]. Considering that lncRNA has an important role in pathological development of colorectal cancer and acts as a kind of transcriptional product, we hypothesize that it may also participate in the process of accommodating the hypoxia environment. In the present study, we applied high-throughput RNA sequencing (RNA-seq) to identify the differences of lncRNA and mRNA expression profiles between normoxic and hypoxic conditions in a colorectal cancer cell line. Multiple layers of bioinformatic analysis of the candidate lncRNAs and mRNAs identified several novel molecules that may be involved in the hypoxia process.

Material and Methods

Cell culture

The human colorectal cancer cell line SW480 were purchased from the American Type Culture Collection (ATCC). The cells

were cultured in high glucose Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) at 37°C in a humidified 5% CO₂ atmosphere. For hypoxic treatment, SW480 cells were cultured in a humidified hypoxic chamber gassed with 1% O₂, 5% CO₂, and 94% N₂.

Total RNA extraction and sequencing

Total RNA was extracted with TRIzol Reagent (Invitrogen, USA) following the manufacturer's instructions. The RNA quality was assessed with NanoDrop ND-1000 (Thermo Fisher Scientific, USA) and RNA integrity was identified using standard denaturing agarose gel electrophoresis. Total RNA was further purified with the RNA Clean XP Kit (Beckman Coulter, USA). Illumina HiSeq 2000/2500 (Illumina, USA) was used to conduct RNA-seq [10]. Three biological replicates were performed. Expression levels of genes and transcripts were compared using edgeR. Differentially expressed lncRNAs and mRNAs were considered significant upon meeting the following thresholds: False Discovery Rate (FDR) <0.01, *P* value <0.05, and fold-change >2.

Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation

To identify characteristic biological attributes of the differentially expressed mRNAs, we conducted the Gene Ontology (GO) analysis. To identify hypoxia-related signaling pathways which were associated with differentially expressed mRNAs, we accessed the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The GO enrichment and KEGG pathway analysis were performed using clusterProfiler package [11]. Significant correlations between mRNAs and their associated functions and/or pathways were considered significant upon the threshold *P*<0.05.

Results

Differentially expressed mRNAs and lncRNAs

Expression levels of mRNAs and lncRNAs were statistically significantly expression between SW480 cells under hypoxia (SW480_H) and SW480 cells under normoxia (SW480_N) (*P*<0.05; fold-change >2). Base on the RNA-seq data, we found that 77 lncRNAs and 1327 mRNAs were differentially expressed between SW480_H and SW480_N. Among them, 50 lncRNAs and 669 mRNAs were upregulated and 22 lncRNAs and 658 mRNAs were downregulated (Figure 1A, 1B). Upon a threshold of fold change of more than 2, Figure 1C and 1D show the upregulated and downregulated lncRNAs and mRNAs. The top 10 differentially expressed lncRNAs regulated by hypoxia are listed in Table 1. In the upregulated lncRNAs, the maximum fold change was 9.994, which belonged to RP11-2B6.3. Among the downregulated lncRNAs, RP11-126K1.2 possessed the

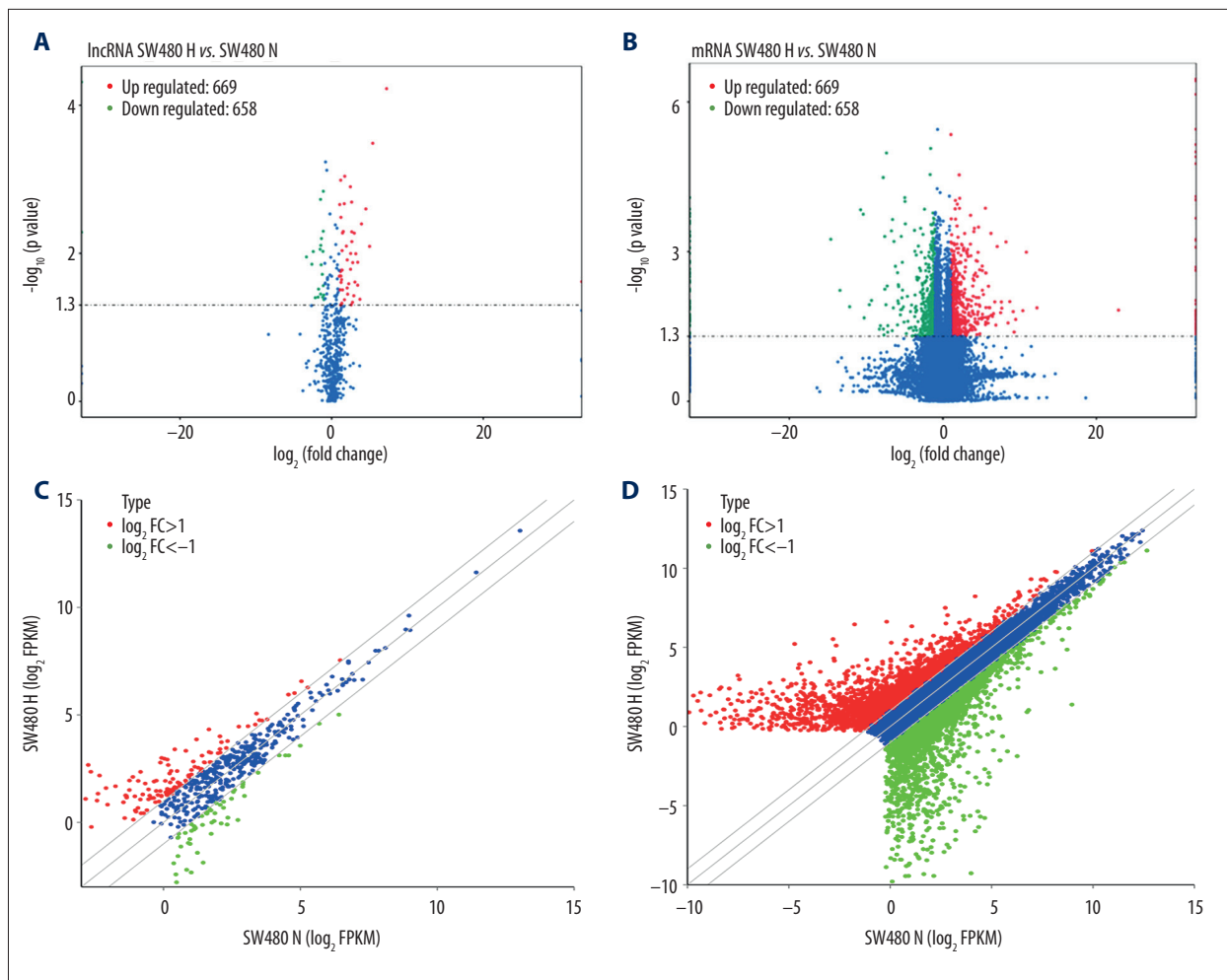


Figure 1. The profiles of differentially expressed lncRNAs and mRNAs in normoxic and hypoxic conditions of colorectal cancer cell line SW480. (A) Differentially expressed lncRNAs and (B) differentially expressed mRNAs were analyzed using volcano plot. “Red” indicates upregulated expression, and “green” indicates downregulated expression. The scatter plot is a visualization method used for showing differentially expressed (C) lncRNAs and (D) mRNAs when fold change was more than twice.

maximum fold change, which was 152.316. Hierarchical clustering analysis was adopted to arrange samples into groups based on lncRNA and mRNA expression levels, which we then used to infer the relationship between samples (Figure 2A, 2B).

Function analysis of differentially expressed genes

GO analysis was applied to analyze the important functions of differentially expressed mRNAs according to the GO database. We found that in the upregulated mRNAs from SW480_H (Figure 3A), the highest enriched GO terms were the cellular macromolecule metabolic process (biological process), the nucleus (cellular component), and the DNA binding (molecular function). Also, in the downregulated mRNAs (Figure 3B), the highest enriched GO terms were the chromatin silencing (biological process), the nucleosome (cellular component), and the protein heterodimerization activity (molecular function).

Pathway analysis of differentially expressed genes

Pathway analysis indicated that 14 pathways and 19 pathways were significant enrichment among the upregulated mRNAs and downregulated mRNAs respectively (Figure 4, [Supplementary Tables 1 and 2, available on request from authors]). We found that the most enriched pathway was “Pathways in cancer” (P value < 0.01), which was associated with 22 differentially expressed genes in the upregulated mRNAs (Figure 4A). In the downregulated mRNAs, the most enriched pathway was “Systemic lupus erythematosus” (P value < 0.01), which was associated with 39 differentially expressed genes (Figure 4B). Among the enrichment pathways, many pathways were linked to cancer, such as “TNF signaling pathway” (associated with 8 genes), “Focal adhesion” (associated with 5 genes), “Viral carcinogenesis” (associated with 23 genes), “Colorectal cancer” (associated with 4 genes), “Transcriptional misregulation

Table 1. Top 10 differentially expressed lncRNAs regulated by hypoxia.

Hypoxia induced lncRNAs			Hypoxia inhibited lncRNAs		
Gene name	Fold change	P value	Gene name	Fold change	P value
RP11-2B6.3	9.994	0.011190355	RP11-126K1.2	152.316	5.97E-05
AC011747.6	6.093	0.014048931	MIR210HG	42.869	0.000326344
LINC00476	5.365	0.009459508	RP3-438O4.4	32.174	0.008059527
MAPKAPK5-AS1	4.458	0.039781437	LINC01119	22.901	0.002514434
VPS9D1-AS1	3.447	0.039067659	RP11-74J13.8	15.337	0.004009094
SNHG1	3.309	0.030433628	LUCAT1	13.269	0.041786033
RP11-119J18.1	2.854	0.008812985	NIFK-AS1	10.875	0.013215553
HOXB-AS3	2.754	0.001870278	CTB-22K21.2	10.821	0.026955415
AC068580.5	2.731	0.035547274	TM4SF1-AS1	9.114	0.010134287
SNHG25	2.693	0.00781051	RP11-798M19.6	8.405	0.024455481

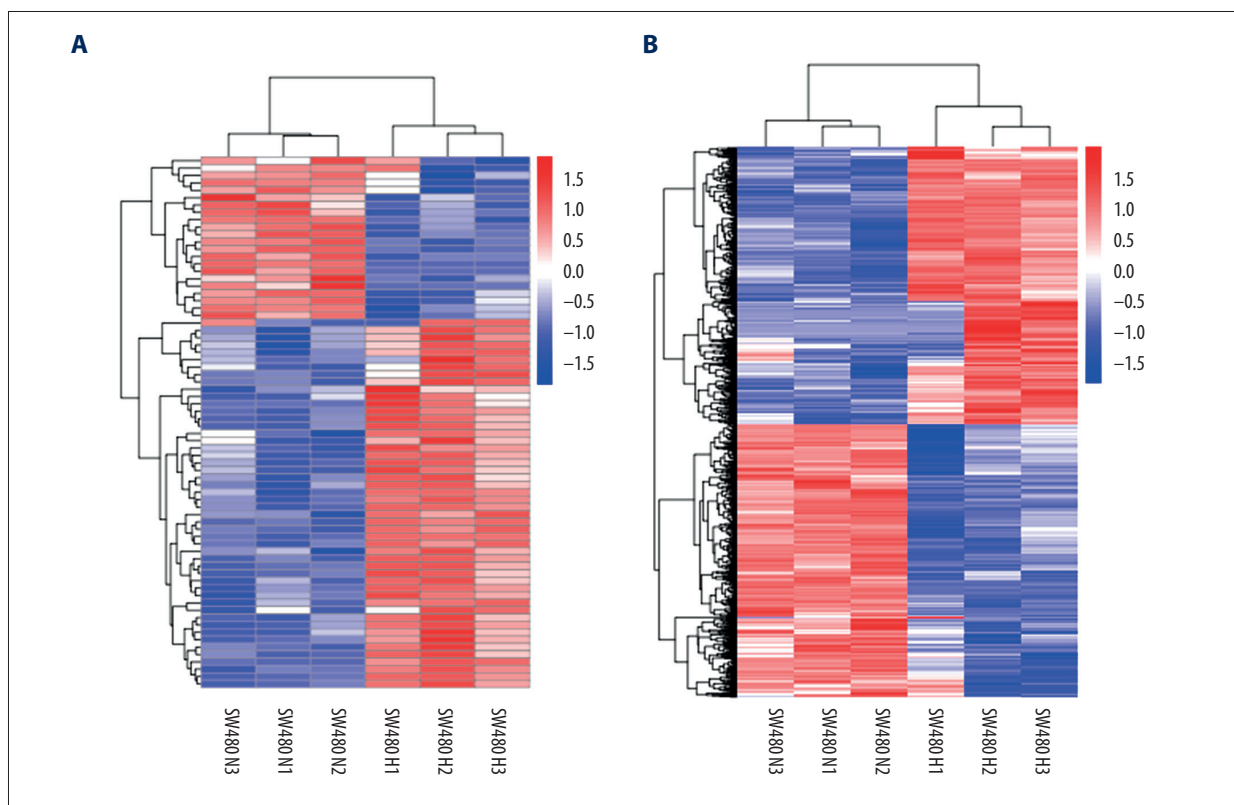


Figure 2. The differentially expressed profiles between normoxic and hypoxic conditions. Hierarchical clustering of (A) lncRNA and (B) mRNA were used for analysis of gene expression data. “Red” indicates high relative expression, and “green” indicates low relative expression.

in cancer” (associated with 15 genes) and “Alcoholism” (associated with 40 genes).

Discussion

Colorectal cancer remains a fatal malignancy worldwide. Because of the emergence of new treatment options and early diagnosis, the overall patient outcome for colorectal cancer has become better. However, about 70% to 80% of colorectal cancer

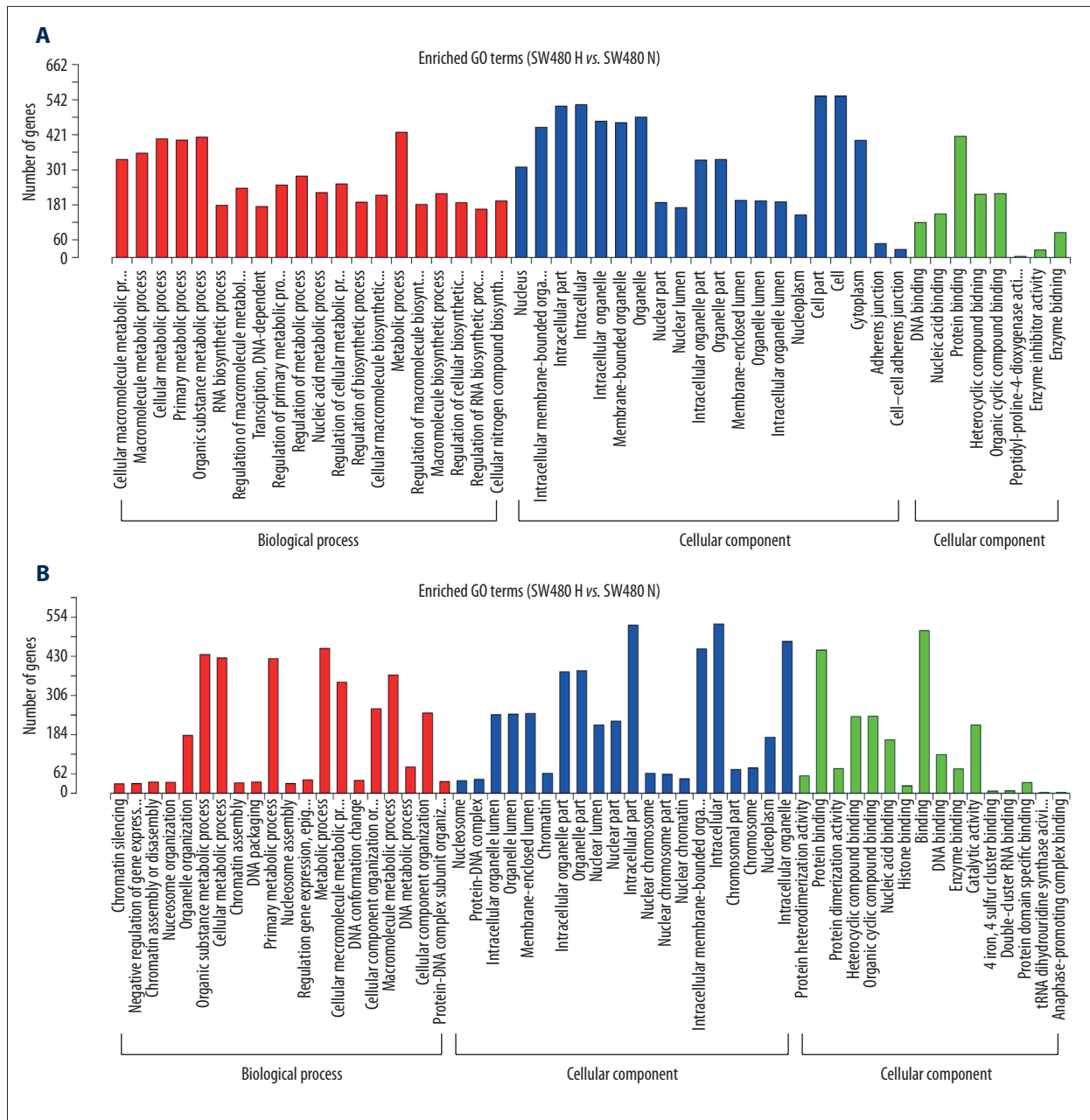


Figure 3. Gene Ontology (GO) analysis of the differentially expressed mRNAs from our dataset. **(A)** Shows the result of GO analysis of upregulated mRNAs from our dataset. **(B)** Shows the result of GO analysis of downregulated mRNAs.

patients will develop metastasis or recurrence and the cure rate is still around ~20% [12]. This indicates that alternative treatment options or biomarkers that will halt or reduce the tumor growth should be introduced in treatment protocols. A prerequisite for tumor growth beyond a few millimeters is angiogenesis, which plays a key role in tumor growth and metastasis. Among the several factors that promote angiogenesis, hypoxic environment is considered to be an important predisposing factor for angiogenesis, such as HIF gene family could transcribe the downstream genes for angiogenesis [13]. Therefore,

exploring the effects of hypoxic conditions on colorectal cancer will help us discover new treatments and new information about pathogenesis.

In the present study, a group of lncRNAs and mRNAs were identified that possessed different expression profiles under hypoxia or normoxia experimental conditions. lncRNAs have been investigated in colorectal cancer, and several lncRNAs have been found related to hypoxia-mediated metastasis [14], growth [15], or epithelial-mesenchymal transition [16]. Here, high-throughput

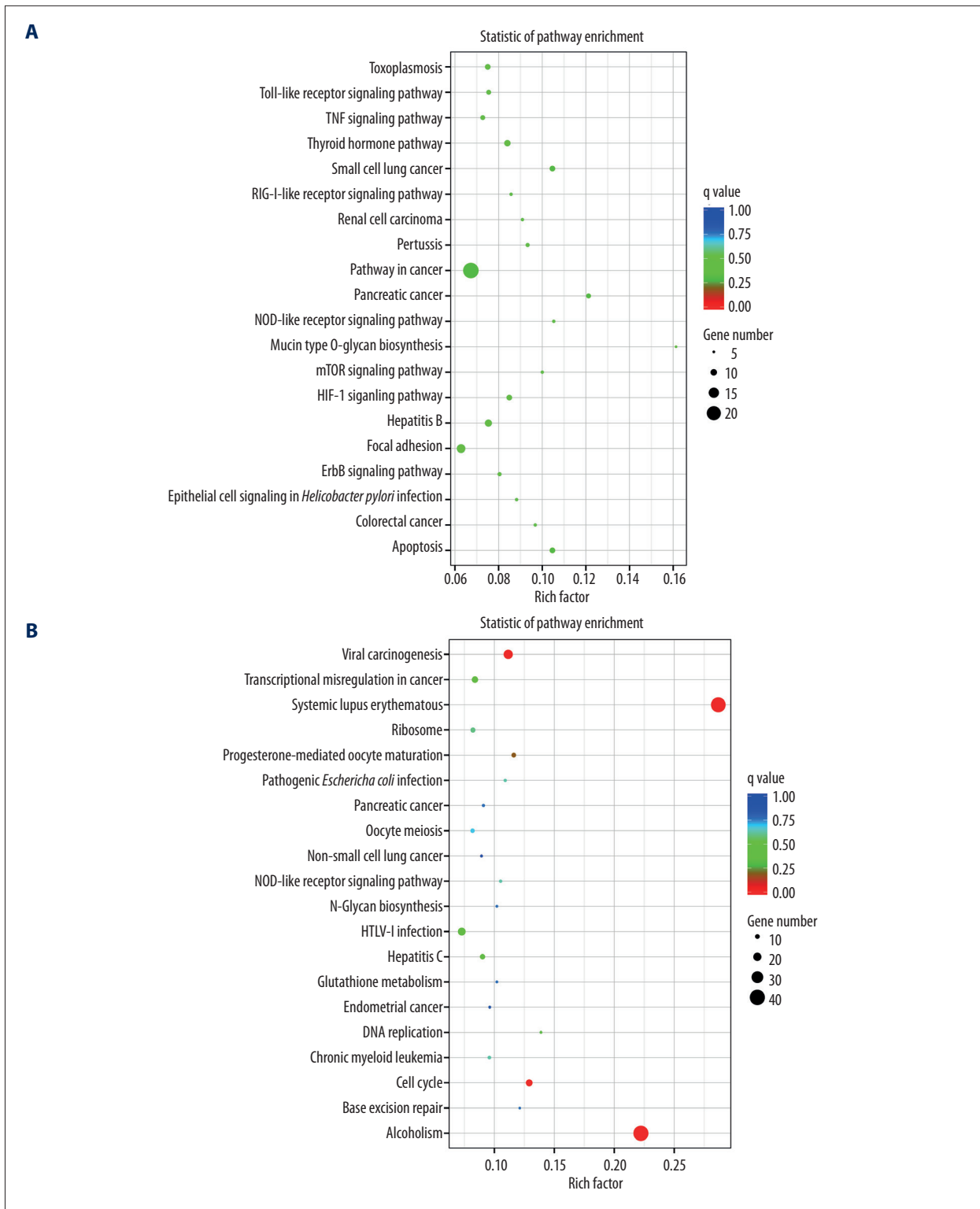


Figure 4. Pathway analysis of the differentially expressed mRNAs from our dataset. **(A)** Shows the result of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of downregulated mRNAs from our dataset. **(B)** Shows the result of KEGG pathway analysis of upregulated mRNAs.

RNA-seq analysis shown that 77 lncRNAs had a different expression pattern under hypoxia and normoxia conditions. We discovered several novel lncRNAs that had not been reported before in colorectal cancer including RP11-126K1.2, RP3-438O4.4, LINC01119, CTB-22K21.2, RP11-798M19.6, and RP11-2B6.3, which were among the top 10 differentially expressed lncRNAs regulated by hypoxia. In addition, several lncRNAs had been reported to be related to hypoxia, such as MIR210HG [17,18] and LUCAT1 [15]. He et al. found that MIR210HG participated in the progression of colorectal adenocarcinoma by regulating hypoxia, and which might function through a regulatory network with MIR210 and RASSF7 [19]. The mechanism of how lncRNAs response to hypoxia in colorectal cancer has not been fully elucidated, despite several studies having been published. Therefore, further studies on lncRNA expression profiles will help us to understand the mechanism of colorectal cancer response to hypoxia and enable development of novel therapeutic strategies and biomarkers.

Through the GO and KEGG pathway analysis, we identified the biological functions and pathways enriched among the differentially expressed mRNAs. GO analysis showed that the differentially expressed genes were mostly involved in basic metabolic processes and gene transcription processes, which may suggest that the differentially expressed lncRNAs could regulate the hypoxia response of colorectal cancer cells via regulating the expression of these genes. Pathway analysis revealed

that many pathways were linked to cancer, such as “TNF signaling pathway”, “Viral carcinogenesis”, “Colorectal cancer”, “Transcriptional misregulation in cancer” and “Alcoholism”. This may suggest that the differently expressed lncRNAs may regulate the hypoxia response of colorectal cancer cells through these canonic pathways.

Conclusions

In this study, we profiled differentially expressed lncRNAs and mRNAs in normoxic and hypoxic conditions of colorectal cancer cells. Further information on the differentially expressed lncRNAs and mRNA were studied. Furthermore, our study revealed that several novel lncRNAs may regulate the response of colorectal cancer cells to hypoxia by altering the expression of genes involved several classical pathways, like “TNF signaling pathway”, “Viral carcinogenesis” and “Colorectal cancer”. Meanwhile, further studies of the possible mechanism are ongoing. Our study indicated that lncRNAs play critical roles in the hypoxia response process of colorectal cancer, which may help to explore the mechanism of initial events in colorectal carcinogenesis.

Conflicts of interest

None.

References:

1. Torre LA, Bray F, Siegel RL et al: Global cancer statistics, 2012. *Cancer J Clin*, 2015; 65(2): 87–108
2. Ponting CP, Oliver PL, Reik W: Evolution and functions of long noncoding RNAs. *Cell*, 2009; 136(4): 629–41
3. Lv L, Wei M, Lin P et al: Integrated mRNA and lncRNA expression profiling for exploring metastatic biomarkers of human intrahepatic cholangiocarcinoma. *Am J Cancer Res*, 2017; 7(3): 688–99
4. Harris AL: Hypoxia – a key regulatory factor in tumour growth. *Nat Rev Cancer*, 2002; 2(1): 38–47
5. Greenhough A, Bagley C, Heesom KJ et al: Cancer cell adaptation to hypoxia involves a HIF-GPRC5A-YAP axis. *EMBO Mol Med*, 2018; 10(11): pii: e8699
6. Li H, Rokavec M, Jiang L, Horst D, Hermeking H: Antagonistic effects of p53 and HIF1A on microRNA-34a regulation of PPP1R11 and STAT3 and hypoxia-induced epithelial to mesenchymal transition in colorectal cancer cells. *Gastroenterology*, 2017; 153(2): 505–20
7. Deschoemaeker S, Di Conza G, Lilla S et al: PHD1 regulates p53-mediated colorectal cancer chemoresistance. *EMBO Mol Med*, 2015; 7(10): 1350–65
8. Wilson WR, Hay MP: Targeting hypoxia in cancer therapy. *Nat Rev Cancer*, 2011; 11(6): 393–410
9. Kaidi A, Williams AC, Paraskeva C: Interaction between beta-catenin and HIF-1 promotes cellular adaptation to hypoxia. *Nat Cell Biol*, 2007; 9(2): 210–17
10. Zhang Z, Jia H, Gu T et al: RNA sequencing and bioinformatics analysis of the long noncoding RNA-mRNA network in colorectal cancer. *J Cell Biochem*, 2018; 119(12): 9957–66
11. Yu G, Wang LG, Han Y, He QY: ClusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS*, 2012; 16(5): 284–87
12. Huang S, Tan X, Huang Z et al: MicroRNA biomarkers in colorectal cancer liver metastasis. *J Cancer*, 2018; 9(21): 3867–73
13. Semenza GL: Expression of hypoxia-inducible factor 1: Mechanisms and consequences. *Biochem Pharmacol*, 2000; 59(1): 47–53
14. Yang F, Huo XS, Yuan SX et al: Repression of the long noncoding RNA-LET by histone deacetylase 3 contributes to hypoxia-mediated metastasis. *Mol Cell*, 2013; 49(6): 1083–96
15. Nishizawa Y, Konno M, Asai A et al: Hypoxia stimulates the cytoplasmic localization of oncogenic long noncoding RNA LINC00152 in colorectal cancer. *Int J Oncol*, 2018; 52(2): 453–60
16. Zhang S, Wang W, Liu G et al: Long non-coding RNA HOTTIP promotes hypoxia-induced epithelial-mesenchymal transition of malignant glioma by regulating the miR-101/ZEB1 axis. *Biomed Pharmacother*, 2017; 95: 711–20
17. Voellenkle C, Garcia-Manteiga JM, Pedrotti S et al: Implication of long non-coding RNAs in the endothelial cell response to hypoxia revealed by RNA-sequencing. *Sci Rep*, 2016; 6: 24141
18. Mimura I, Hirakawa Y, Kanki Y et al: Novel lncRNA regulated by HIF-1 inhibits apoptotic cell death in the renal tubular epithelial cells under hypoxia. *Physiol Rep*, 2017; 5(8): pii: e13203
19. He Z, Dang J, Song A et al: Identification of LINC01234 and MIR210HG as novel prognostic signature for colorectal adenocarcinoma. *J Cell Physiol*, 2019; 234(5): 6769–77