

MicroRNA expression profiling in the colorectal normal-adenoma-carcinoma transition

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Abstract. Colorectal adenoma is a major precursor to colorectal cancer. Investigating the alteration of microRNA (miRNA/miR) expression during the progression from normal colorectal tissue to adenoma, and finally to colorectal carcinoma may aid our understanding of the biological mechanisms of colorectal tumorigenesis. In the present study, the miRNA expression profiles of normal colorectal tissue, adenoma and colorectal carcinoma from 6 patients were evaluated using miRNA-sequencing. A total of 334 miRNAs were identified as differentially expressed. It was revealed that 34 miRNAs were upregulated in all 6 patients, including miR-135b-5p, miR-18a-5p and miR-29b-3p, and 28 miRNAs were down-regulated, including miR-1-3p, miR-338-3p and miR-218-5p. Using bioinformatic analysis, the potential target genes of these 62 miRNAs were predicted and found to be enriched in 'transcription, DNA-dependent (GO:0006351)', 'signal transduction (GO:0007165)', 'small molecule metabolic process (GO:0044281)', 'PI3K/AKT signaling pathway (path ID:04151)' and 'MAPK signaling pathway (path ID:04010)'. The miRNA expression profiles identified in the present study may extend our understanding of the molecular mechanisms underlying colorectal tumorigenesis and promote novel perspectives for prevention, diagnosis and treatment.

Introduction

As one of the most common forms of cancer, colorectal cancer is the third most frequent cause of cancer-related mortality worldwide, accounting for ~1.36 million new cases and 0.69 million estimated mortalities each year (1). Colorectal adenoma is a major precursor to colorectal cancer, with an

estimated 5% of adenomas developing into carcinomas (2,3). MicroRNAs (miRNAs/miRs) are small, non-coding RNAs that post-transcriptionally modulate the stability of messenger RNAs to regulate gene expression and serve crucial roles in colorectal tumorigenesis (4). Therefore, clarifying the alteration of miRNA expression profiles in the progression from normal colorectal tissue to adenoma, and finally to carcinoma, is essential for an improved understanding of the biological mechanisms of colorectal tumorigenesis.

Global expression analysis based on high-throughput data has become a powerful technique in miRNA analysis (5). In the present study, using miRNA-sequencing, the miRNA expression profiles in matching normal colorectal tissue, adenoma and carcinoma samples from patients were detected. Critical miRNAs that may be involved in cancer progression were investigated, and their biological functions were assessed through comprehensive bioinformatic analysis. The present results may facilitate a better understanding of the roles of miRNAs in colorectal tumorigenesis and promote novel strategies for the prevention and treatment of colorectal carcinoma.

Materials and methods

Ethics statements and patient selection. The present study was approved by The Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China). Written informed consent was obtained from the 6 male patients with colorectal adenoma and carcinoma and three adjacent samples were collected using colonoscopy from each patient with normal mucosa, adenoma and carcinoma between January 2014 and July 2014. The age of the patients ranged between 49-64 years (mean age \pm standard deviation, 57.67 \pm 6.83 years).

RNA isolation. Total RNA was extracted from tissue samples using the RNeasy Pure kit (For Tissue) (Qiagen Biotech Co., Ltd.) following the manufacturer's protocol. The concentration and purity of RNA samples were determined by absorbance detection and electrophoresis.

miRNA-sequencing. The sequencing libraries were constructed according to the protocol for the Illumina small RNA sample preparation kit (6). Sequencing was performed on the Illumina HiSeq 2000 sequencer (Illumina, Inc.). Library construction and sequencing were performed at the Geneng Biotech

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Co., Ltd. miRNA expression was analyzed by miRdeep version 2.0.0.7 (6).

Screening for differentially expressed miRNAs. ANOVA and least significant difference post hoc test were performed in GraphPad Prism version 5 (GraphPad Software, Inc.) to compare the mean differences of the expression among the three groups in normal colorectal tissues, colorectal adenomas and tumor tissues. miRNAs with $P < 0.05$ and fold-change > 2 were considered significantly altered. miRNAs among all 6 patients that were consistently and significantly increased or decreased in adenoma and cancer were identified using an FDR cutoff value of 0.05 and absolute log₂-fold change greater than 1, and selected for the next stage of analysis. Hierarchical clustering was performed to further sort the selected differentially expressed miRNAs with similar expression patterns, using Gene Cluster 3.0 with the following settings: Distance metric, Pearson correlation; linkage rule, average linkage (bonsai.hgc.jp/~mdehoo/software/cluster/software.htm).

Prediction of miRNA target genes. The target genes of these miRNAs were predicted using TargetScan (www.targetscan.org) (7) and miRanda (www.microrna.org/microrna/home.do) (8) databases. The overlapping predictions obtained from the two databases were considered as potential target genes.

Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) analyses. GO and KEGG analyses were applied to analyze the main functions of the target genes and identify the significant pathways. The GO (geneontology.org) (9) and KEGG (www.genome.jp/kegg) (10) databases were used as previously reported (5,11). Enrichment analysis provided a measure of the significance of the GO and KEGG pathways. A threshold of $P < 0.05$ indicated statistical significance.

Results

Identification of differentially expressed miRNAs. Microarray analysis revealed 334 differentially expressed miRNAs in one normal colorectal, adenoma and carcinoma group compared with the other two groups. Among them, 34 miRNAs were consistently upregulated in tissue undergoing colorectal adenoma-carcinoma transition, including miR-135b-5p, miR-18a-5p and miRNA-29b-3p (the three with the largest fold changes), while 28 miRNAs were consistently downregulated, including miR-1-3p, miR-338-3p and miR-218-5p (the three with the largest fold changes) (Fig. 1). The results of hierarchical cluster analysis of the miRNAs with the largest up and downregulated changes are shown in Fig. 2.

The target genes of these 62 miRNAs were predicted using the miRNA target site databases TargetScan and miRanda, and the overlapping results are listed in Table SI, including 3,715 genes.

Enriched GO terms. Using GO analysis, it was found that the potential target genes of the consistently upregulated miRNAs were significantly enriched in 561 GO terms, while which of consistently downregulated miRNAs were

significantly enriched in 475 GO terms. The potential targets of differentially expressed miRNAs were mainly enriched in transcription regulation, signaling and metabolism of biological process, extracellular matrix (ECM), and signal transducer activity of molecular function during colorectal tumorigenesis, such as 'transcription, DNA-dependent (GO:0006351)', 'signal transduction (GO:0007165)', 'small molecule metabolic process (GO:0044281)', 'apoptotic process (GO:0006915)', 'cell adhesion (GO:0007155)' and 'protein transport (GO:0015031)', consistent with the proliferative and invasive capacity of colorectal cancer cells (Fig. 3). In addition, other GO functions associated with target genes included 'activation of MAPK activity (GO:0000187)', 'cell cycle (GO:0007049)', 'cell-cell signaling (GO:0007267)', 'cell differentiation (GO:0030154)' and 'protein ubiquitination (GO:0016567)'.

Significant KEGG pathways. Using KEGG analysis, it was found that the potential target genes of upregulated miRNAs were significantly enriched in 135 signaling pathways, while those of downregulated miRNAs were significantly enriched in 106 signaling pathways. The majority of these signaling pathways overlapped. The results revealed that potential target genes of the upregulated and downregulated miRNAs are involved in the 'MAPK signaling pathway', which is consistent with the GO analysis (Fig. 4). Target genes were also significantly enriched in the 'PI3K/AKT signaling pathway', 'focal adhesion' and the 'Hippo signaling pathway'.

Discussion

In the present study, high throughput screening of miRNA expression profiles was performed by sequencing miRNA from adjacent non-tumor, colorectal adenoma and carcinoma tissue samples from the same patient. Careful screening revealed 334 miRNAs as significantly differentially expressed in the three tissues types. These differentially expressed miRNAs may be promising potential diagnostic markers for the progression of colorectal cancer. In support of these findings, a study by Slattery *et al* (12) also identified ~600 differentially expressed miRNAs in colorectal carcinoma progression using an Agilent microarray platform (12).

In the present study, the potential function of the 62 miRNAs which were consistently upregulated or downregulated during the progression from normal to adenoma, and subsequently to carcinoma, was investigated. It was revealed that miR-135b-5p, miR-18a-5p and miR-29b-3p were consistently upregulated during this transition. Several studies have confirmed the overexpression of miR-135b-5p in colorectal tumors (13-15). Valeri *et al* (13) reported that miR-135b-5p overexpression was triggered in mice and humans by loss of adenomatous polyposis coli, PTEN/PI3K pathway deregulation and overexpression of proto-oncogene tyrosine-protein kinase Src, and it was also demonstrated that miR-135b-5p promoted tumor transformation and progression. Zhang *et al* (14) and Liu *et al* (16) reported that miR-135b-5p acted as an upstream factor of the PI3K/AKT pathway, while the results presented in this study demonstrated that the PI3K/AKT pathway was enriched for targets

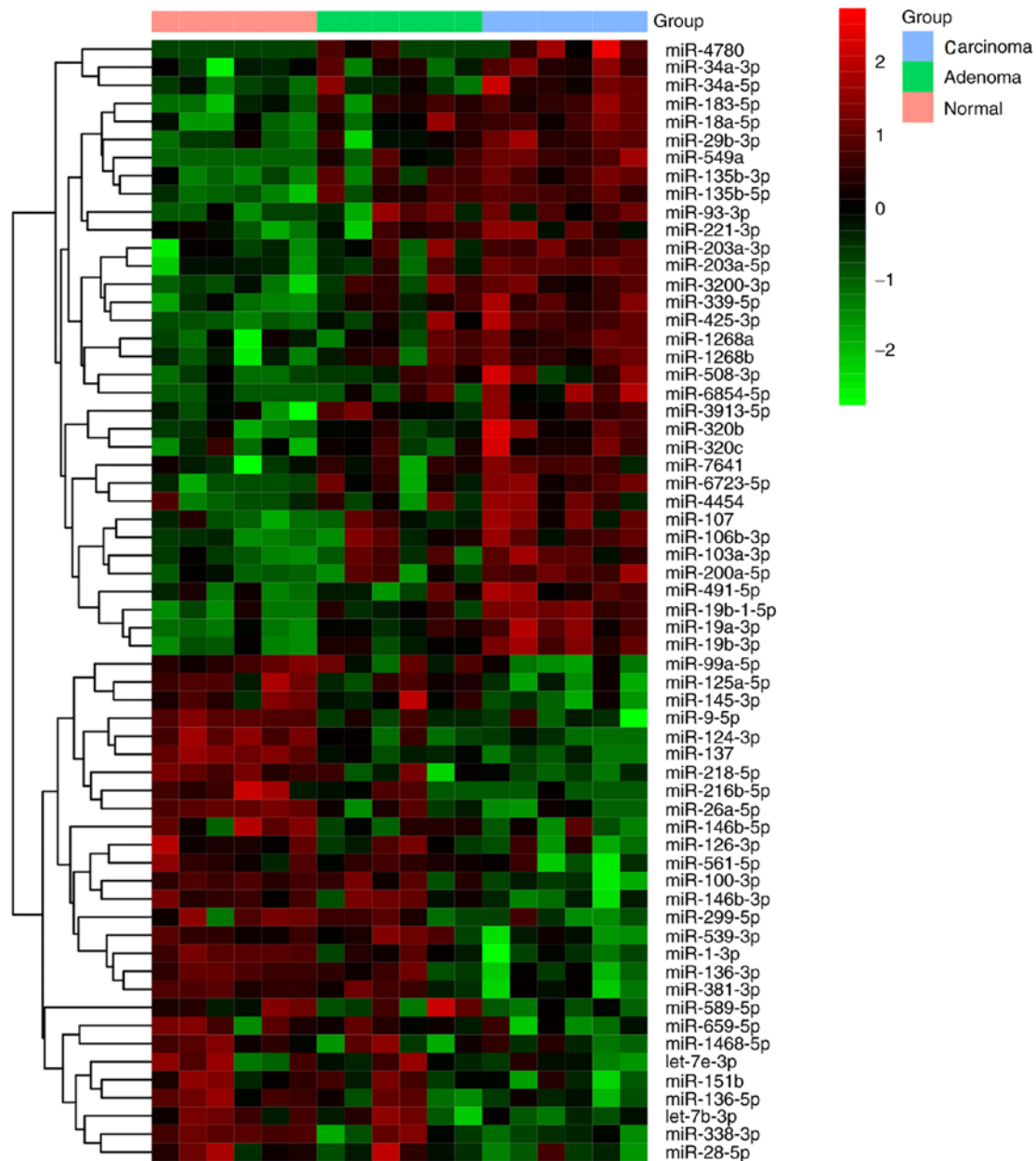


Figure 1. Cluster analysis of the 34 consistently upregulated miRNAs and the 28 consistently downregulated miRNAs in normal colorectal tissue, adenoma and carcinoma samples. Red represents upregulation of miRNAs, and green represents downregulation of miRNAs. miRNA/miR, microRNA.

of differentially expressed miRNAs. Yau *et al* (17) reported that miR-18a-5p expression was significantly increased in tumor tissues and stool samples of patients with colorectal carcinoma, and that it may serve as a potential biomarker for a non-invasive diagnosis of this cancer. In the present study, miR-29b-3p was significantly upregulated in colorectal tumors. However, Inoue *et al* (18) reported that miR-29b-3p expression was significantly downregulated in tumor tissue compared with normal mucosa, and that it was an independent positive prognostic factor of disease-free survival. Wang *et al* (19) reported that miR-29b-3p suppressed tumor growth and metastasis in colorectal cancer by downregulating T-cell lymphoma invasion and metastasis 1 expression and inhibiting epithelial to mesenchymal transition. Therefore, further study is required to clarify the impact of altered miR-29b-3p expression in colorectal tumors.

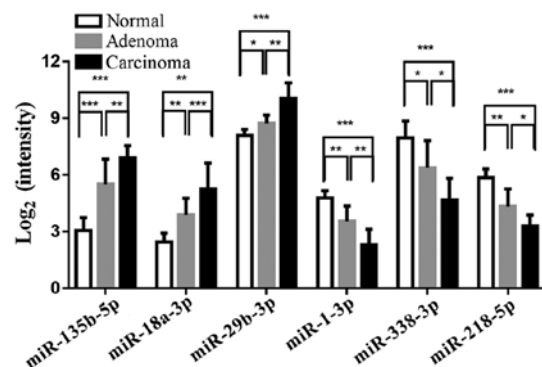


Figure 2. Expression of the three most upregulated miRNAs (miR-135b-5p, miR-18a-5p and miR-29b-3p) and the three most downregulated miRNAs (miR-1-3p, miR-338-3p and miR-218-5p) in the colorectal normal-adenoma-carcinoma transition, as calculated from miRNA sequencing data. miRNA/miR, microRNA. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

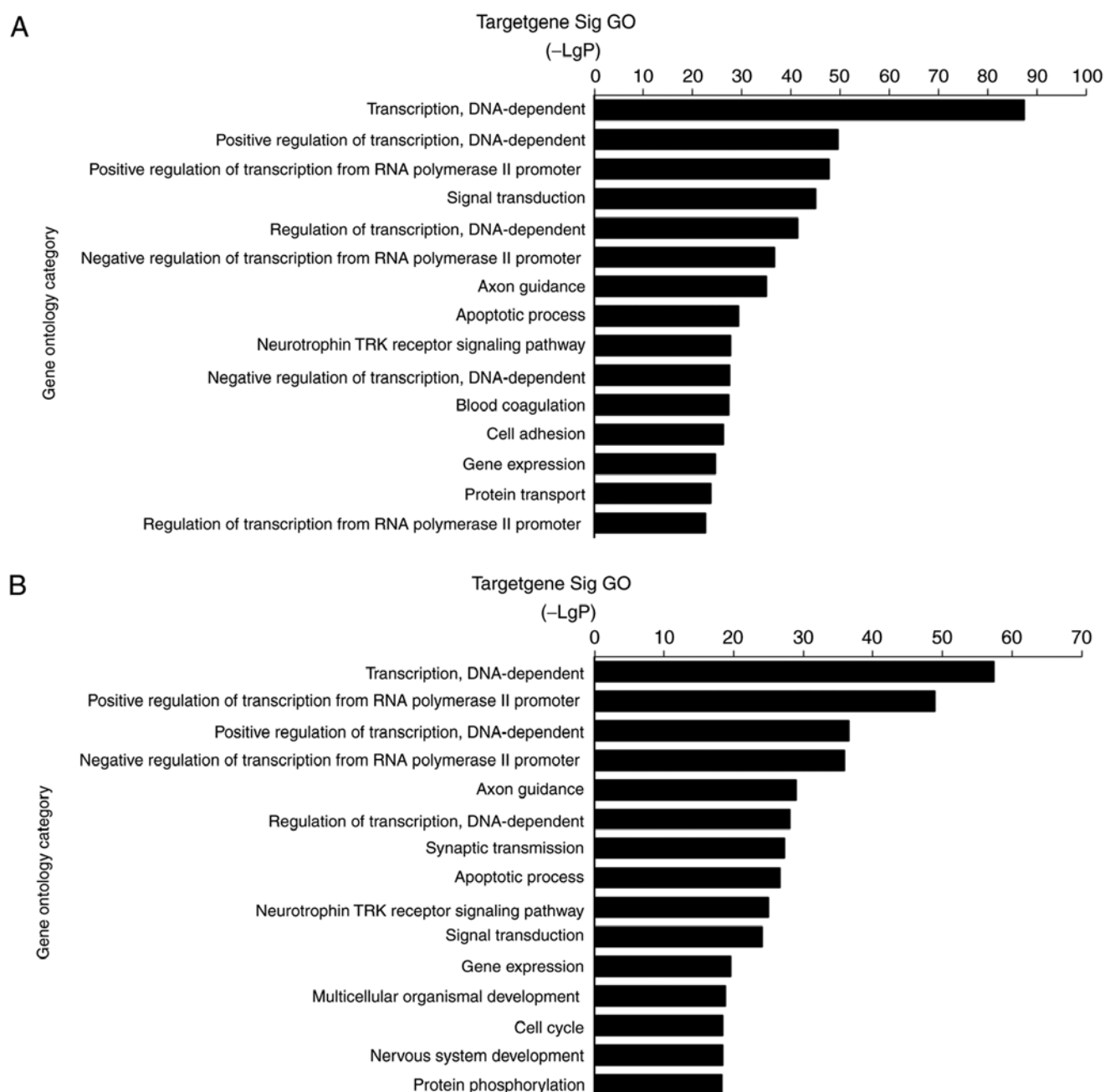


Figure 3. GO analysis. (A) The 15 most significantly upregulated GO terms, and (B) 15 most significantly downregulated GO terms in the colorectal normal-adenoma-carcinoma transition. GO, Gene Ontology; Sig, significant; -LgP, $-\log_{10}$ (P-value).

The present study also revealed that miR-1-3p, miR-338-3p and miR-218-5p were significantly downregulated throughout the transition from normal tissue to adenoma and then carcinoma. A recent meta-analysis of The Cancer Genome Atlas and Gene Expression Omnibus data performed by Wang *et al* (20) demonstrated significant miR-1-3p downregulation in colorectal cancer. Specifically, miR-1-3p was found to suppress tumor proliferation via the mothers against decapentaplegic homolog 3, hypoxia-inducible factor 1, and MAPK and PI3K pathways (21,22). Sun *et al* (23) reported that miR-338-3p expression was reduced in colorectal cancer and was negatively correlated with advanced TNM stage and local invasion. It was also reported that miRNA-338-3p suppressed cell proliferation of human colorectal carcinoma by targeting the smoothened

gene (24). Furthermore, several studies reported miR-218-5p downregulation in colorectal cancer, which exerted its tumor suppressor effects via the PI3K/AKT pathway, diphthamide biosynthesis 1 and metastasis-associated in colon cancer 1, among others (25-28).

Based on the present results, the predicted target genes of differentially expressed miRNAs were enriched in cell cycle, cell adhesion and the PI3K and MAPK signaling pathways. The majority of these GO terms and KEGG pathways have been demonstrated to serve significant roles in colorectal tumorigenesis. Sun *et al* (29) found that the MAPK pathway was associated with cell proliferation, differentiation, migration, senescence and apoptosis in colorectal carcinoma. Bogaert and Prenen (30) concluded the PI3K/AKT, WNT, p53, receptor tyrosine

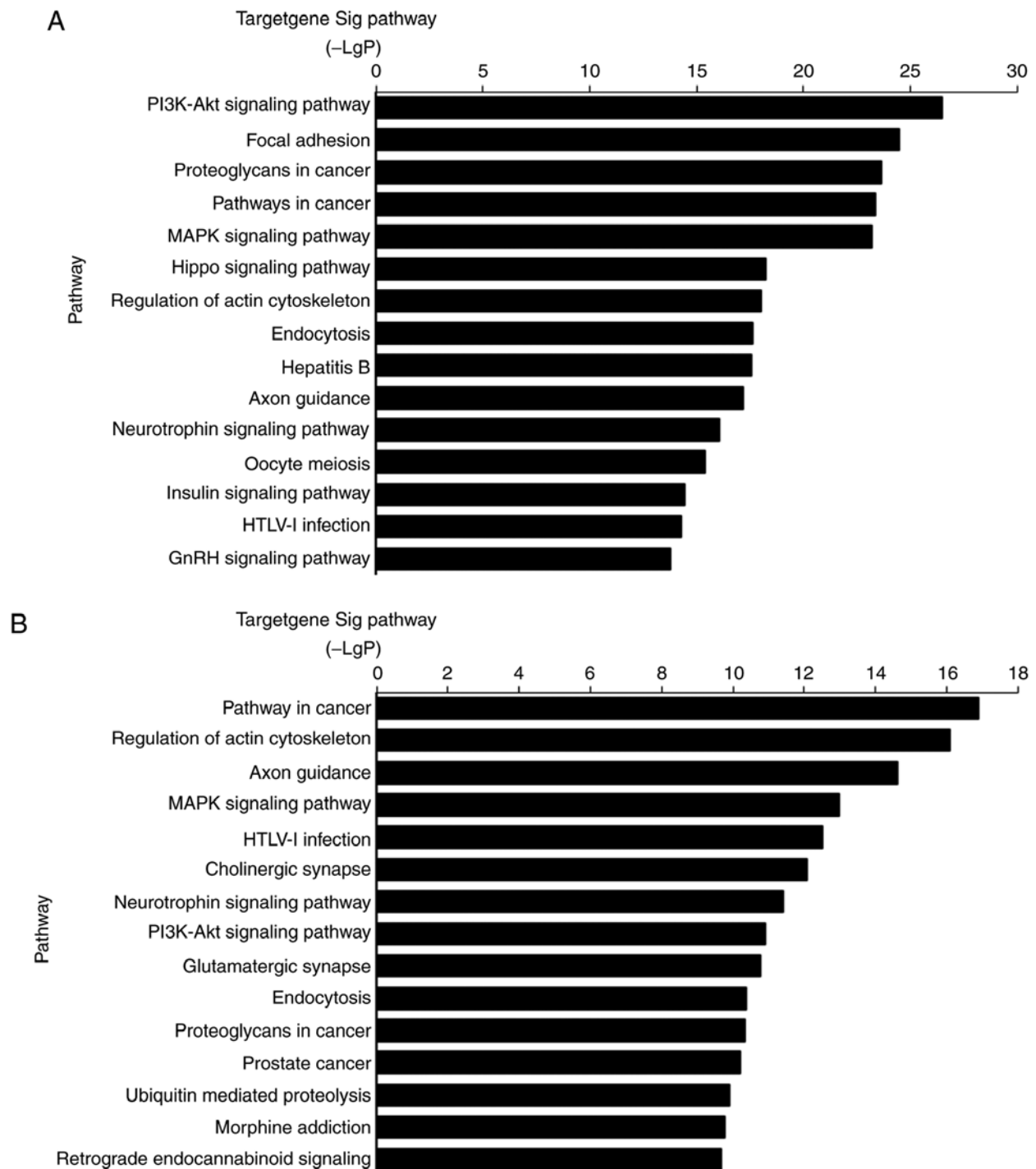


Figure 4. KEGG pathway analysis. (A) The 15 most significantly upregulated KEGG pathways, and (B) 15 most significantly downregulated KEGG pathways in the colorectal normal-adenoma-carcinoma transition. KEGG, Kyoto Encyclopedia of Genes and Genomes; Sig, significant; -LgP, -log₁₀(P-value).

kinase/RAS GTPase and transforming growth factor β pathways to be the five pathways frequently altered in colorectal cancer. Notably, the predicted target genes identified in the present study were also enriched in the majority of these pathways.

It should be noted that there are several limitations to the present study. First, only samples from 6 patients were used. Second, the expression of miRNAs was not validated by other methods such as quantitative PCR. Third, the majority of the results were based on bioinformatics analysis and require further validation.

In the present study, a high-throughput screening of miRNA expression profiles in colorectal cancer development was performed by sequencing miRNA from adjacent normal, colorectal adenoma and carcinoma tissues. The miRNAs that were consistently and significantly upregulated or down-regulated were selected, and their biological targets were predicted using bioinformatics analysis. These miRNAs may be promising diagnostic biomarkers and potential treatment targets in colorectal carcinoma. These findings should be further validated in future experiments.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available on reasonable request from the corresponding author on reasonable request.

Authors' contributions

LY, YZ and PZ designed the study. JL and YZ wrote the manuscript. JL and SC conducted the experiments and analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Zhongshan Hospital, Fudan University (Shanghai, China) and conducted according to their guidelines and regulations.

Patient consent for publication

All participants provided informed consent.

Competing interests

The authors declare that they have no competing interests.

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