miR-31 promotes tumorigenesis in ulcerative colitis-associated neoplasia via downregulation of SATB2

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Received February 28, 2020; Accepted August 18, 2020

DOI: 10.3892/mmr.2020.11573

Abstract. Ulcerative colitis (UC) features chronic, non-infectious inflammation of the colon. The risk of ulcerative colitis-associated neoplasia (UCAN) increases in direct association with the duration of this disease. Whether miRNAs exert a regulatory effect on the pathogenesis of UCAN has remained to be elucidated. In the present study, differentially expressed genes (DEGs) and microRNAs (miRNAs/miRs) were identified using bioinformatics analysis of Gene Expression Omnibus datasets. Enrichment analyses were performed to determine the function of the DEGs. The target genes of key miRNAs were predicted using miRWalk. Validation of DEGs and miRNAs in patients with UC, UC with low-grade dysplasia and UC with high-grade dysplasia (UC-HGD) was performed using reverse transcription-quantitative PCR analysis. A total of 38 differentially expressed miRNAs and 307 mRNAs were identified from the profiles and miR-31 was validated as being overexpressed in UCAN tissues, particularly in the UC-HGD samples. Furthermore, special AT-rich DNA-binding protein 2 (SATB2) was validated as a target gene of miR-31 and SATB2 expression was negatively correlated with miR-31 expression. Therefore, miR-31 is upregulated in UCAN and it may promote tumorigenesis through downregulation of SATB2.

Introduction

Ulcerative colitis (UC) is a type of inflammatory bowel disease that is characterized by chronic, non-infectious inflammation of the colon (1). To date, the etiology of UC has remained to be fully elucidated. Prolonged duration of active disease is associated with a high risk of UC-associated neoplasia (UCAN) (2). Pathologically, UCAN exhibits a broad range of severity, including low-grade dysplasia (LGD), high-grade dysplasia (HGD) and invasive carcinoma (3). It was reported that ~18% of patients with UC may develop colorectal cancer (CRC) after 30 years, particularly patients with extensive colitis (4). As the detailed mechanisms that participate in the pathogenesis of UCAN remain elusive, associated research is warranted.

MicroRNAs (miRNAs/miRs) are a class of non-coding RNAs that are single-stranded, consist of 19-24 nucleotides and regulate approximately one-third of human genes at the post-transcriptional level via binding to their 3' untranslated region (5). Previous studies demonstrated that miRNAs have a key role in the inflammatory process associated with UC (6,7). Although several studies have proven that miRNAs are involved in CRC (8,9), the pathogenesis of UCAN is not consistent with that of CRC. Furthermore, there is little research on how miRNAs regulate tumorigenesis in UC.

The aim of the present study was to identify differentially expressed miRNAs (DEM) and downstream genes in patients with UCAN using bioinformatics analysis and verify the regulatory effect of candidate miRNAs on their target genes, for the purpose of uncovering an epigenetic regulation involving miRNAs and associated target genes, thereby further contributing to the elucidation of the pathogenesis of UCAN.

Materials and methods

Bioinformatics analysis. Datasets containing miRNA and mRNA expression profiles in the colonic mucosa of patients with UC and UCAN (GSE68306 and GSE37283) were downloaded from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo). Linear Models for Microarray Data (10) was applied to identify differentially expressed genes (DEGs). Fold change >2.0 and P<0.05 were used as the cutoff values. Heatmap, volcano and scatter plots were generated to describe the GEO data visually using ggplot2 and heatmap packages in R. Gene Ontology (GO) (11) and Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway (12) enrichment analyses were performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) online tool (13,14). The miRNA target genes were predicted with the miRWalk tool (mirwalk.umm.uni-heidelberg.de), which integrates the miRNA resources from TargetScan, miRDB and miRTarBase.

Key words: ulcerative colitis, ulcerative colitis-associated neoplasia, miR-31, special AT-rich DNA-binding protein 2, bioinformatics
Patients for validation. A total of 50 patients with UC were enrolled in the present study as the validation cohort. All of the patients were admitted to Tianjin University General Hospital (Tianjin, China) between January 2000 and December 2018. The inclusion criteria were as follows: Patients aged ≥18 and <60 years, with UC of any extent, including proctitis, left-sided colitis and extensive colitis. The exclusion criteria were as follows: Patients with UC who were diagnosed with malignant tumors or other autoimmune diseases, and patients without biopsy as the pathological sample. The Ethics Committee at Tianjin Medical University General Hospital (Tianjin, China) approved the study protocol and informed consent was obtained from all of the enrolled patients. The patients were divided into three groups as follows: UC patients without neoplasia (UC group; n=20), UC patients with low-grade dysplasia (UC-LGD group; n=20) and UC patients with high-grade dysplasia (UC-HGD group; n=10). Colonic mucosa samples were retrieved from archived formalin-fixed paraffin-embedded (FFPE) tissues obtained from these patients by endoscopic biopsy.

RNA extraction and quality check. Total RNA was extracted from the 10-mm sections of the FFPE tissue after deparaffinization as described previously (15) and from the cultured cells. RNA was extracted using the Ambion RecoverAll kit (Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The RNA quality check was performed by Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Complementary DNA (cDNA) was reverse-transcribed from the extracted RNA using the M-MuLV First Strand cDNA Synthesis System (Thermo Fisher Scientific, Inc.) and the miRNA First Strand cDNA Synthesis System (Poly A Tailing; Thermo Fisher Scientific, Inc.). The expression level of candidate miRNAs and mRNAs was determined using the miRNA Quantitation PCR kit (Thermo Fisher Scientific, Inc.) and the SGEexcel FastSYBR Mixture (Thermo Fisher Scientific, Inc.). GAPDH and U6 were used as internal controls. The sequences of the primers used are listed in Table SI. The relative fold changes in miRNA and target gene expression were calculated using the 2^−ΔΔCq method (16).

Cell culture. The human colon cancer cell line SW480 was obtained from the American Type Culture Collection. SW480 cells were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere containing 5% CO2.

Cell transfection. miR-31 mimics and miR negative controls were obtained from Gene Pharma Co. The SW480 cells were seeded in a 6-well plate and at 70-80% confluence, they were transfected with miR-31 mimics and negative controls using the Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol for 48 h at 37°C. The cells were then seeded in 96-well plates at a density of 2x104 per well and cultured at 37°C for 24, 48, 72 or 96 h. A total of 10 µl Cell Counting kit-8 (CCK-8) solution (Sigma-Aldrich; Merck KGaA) was then added to each well. After incubation for 1 h at 37°C, the optical density at 450 nm was detected to evaluate the cell proliferation with Fisherbrand™ accuSkan™ GO ultraviolet/Vis Microplate Spectrophotometer (Thermo Fisher Scientific, Inc.).

Statistical analysis. The experimental results are presented as the mean ± standard error of the mean. The differences in among multiple groups were analyzed using ANOVA (parametric model) followed by Tukey's post-hoc test using GraphPad Prism 7 (GraphPad Software, Inc.). Pearson's correlation analysis was performed to assess the correlation between miRNA and target gene expression. P<0.05 was considered to indicate statistical significance. Heatmaps, volcano and scatter plots, as well as bubble charts, were generated in R.

Results

DEGs. According to the expression profiles of patients with UC and UCAN (GSE37283 dataset), a total of 307 DEGs (P<0.05; fold change >2) were identified. Among these 307 DEGs, 165 were upregulated and 142 were downregulated. The volcano plots were created according to the expression profile (Fig. 1A). A scatter plot (Fig. 1B) and heatmap (Fig. 1C) of mRNA expression levels compared between UC and UCAN groups were also generated, which exhibited an even distribution of gene expression data upon visual examination.

Gene function and pathway enrichment analysis. The functions and pathway enrichment of DEGs were analyzed using DAVID and a false discovery rate <0.05 was used as the cut-off. The top 10 GO terms in which the up- and downregulated DEGs were enriched are listed in Table I. The detailed data of the GO analysis are illustrated in Fig. 2. The result of the KEGG pathway enrichment analysis is presented in Fig. 3. The upregulated DEGs were mainly enriched in complement and coagulation cascades, proteoglycans in cancer, phagosome, natural killer cell-mediated cytotoxicity and the peroxisome proliferator-activated receptor signaling pathway, whereas the downregulated genes were mainly enriched in retinol metabolism, ascorbate and aldarate metabolism, steroid hormone biosynthesis, pentose and glucuronate interconversions, and porphyrin and chlorophyll metabolism.

DEMs and corresponding target genes. Among all miRNAs in the expression profile (GSE68306 dataset), 38 were identified as DEMs (P<0.05; fold change >2; Fig. 4A). A total of 15 DEMs were downregulated, including miR-10b, miR-584, miR-655, miR-453, miR-495, miR-634, miR-615, miR-548, miR-23a, miR-523, miR-455, miR-28, miR-193a and let-7f, while 23 DEMs were upregulated, including miR-106b, miR-34a, miR-31, miR-135b, miR-1974, miR-151-3p, miR-186, miR-423, miR-143, miR-127, miR-1290, miR-381, miR-152, miR-214, miR-374a, miR-140, miR-331, miR-1178, miR-493, miR-1246, miR-141, miR-374b and miR-708. Among these dysregulated miRNAs, miR-31 (17,18), miR-34a (15,19,20) and miR-106b (21) were reported to be differentially expressed in CRC by previous studies. Furthermore, miR-31 was also reported to be dysregulated in UC (8,22). These three miRNAs miR-31,
Figure 1. mRNA expression profiles of patients with UCAN and UC. (A) Volcano plots of mRNA expression in patients with UCAN vs. UC. (B) Scatter plots of mRNA expression compared between UCAN and UC patients. (C) Heat map of mRNAs differentially expressed between patients with UCAN and UC (P<0.05, |fold change| >2). UCAN, UC-associated neoplasia; UC, ulcerative colitis.

Figure 2. GO analyses of differentially expressed mRNAs in patients with UCAN vs. UC. GO terms enriched by the upregulated genes are displayed on the left, while GO terms enriched by the downregulated genes enriched are displayed on the right. GO, Gene Ontology; UCAN, UC-associated neoplasia; UC, ulcerative colitis.
miR-34a and miR-106b, were selected as the candidate miRNAs for further validation. As each miRNA had numerous target genes, there was an overlap among the target genes of these three candidate DEMs and the associations are presented in Fig. 4B.

**Patient characteristics.** The characteristics of the patients enrolled in the present study, including demographic and clinicopathological data and laboratory parameters, are summarized in Table II. There were no statistical differences identified in the average age and sex between the UC, UC-LGD and UC-HGD groups.

Validation of DEMs and corresponding target genes in patient samples. Validation of the three candidate miRNAs (miR-31, GO, Gene Ontology.

Table I. GO terms enriched by the differentially expressed genes in different categories.

<table>
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<tr>
<th>A, Cellular component</th>
<th>GO terms</th>
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<tr>
<td>Upregulated genes</td>
<td>Extracellular region; extracellular exosome; plasma membrane; proteinaceous extracellular matrix</td>
</tr>
<tr>
<td>Downregulated genes</td>
<td>Endoplasmic reticulum membrane; basolateral plasma membrane</td>
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<th>B, Molecular function</th>
<th>GO terms</th>
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<tr>
<td>Upregulated genes</td>
<td>Collagen binding; protein binding</td>
</tr>
<tr>
<td>Downregulated genes</td>
<td>Protein heterodimerization activity; Transferase activity, transferring hexosyl groups; actin filament binding</td>
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<table>
<thead>
<tr>
<th>C, Biological process</th>
<th>GO terms</th>
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<tbody>
<tr>
<td>Upregulated genes</td>
<td>Leukocyte migration; signal transduction; inflammatory response; neutrophil chemotaxis</td>
</tr>
<tr>
<td>Downregulated genes</td>
<td>Negative regulation of glucuronosyltransferase activity; negative regulation of fatty acid metabolic process; flavonoid glucuronidation; flavonoid biosynthetic process; retinoic acid metabolic process</td>
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GO, Gene Ontology.
miR-34a and miR-106b) was performed by RT-qPCR analysis in all samples from patients with UC (n=20), UC-LGD (n=20) and UC-HGD (n=10). The expression of miR-31 among the three groups was significantly different (P<0.01, Fig. 5A). In detail, the expression of miR-31 in patients with UC-HGD was significantly higher compared with that in patients with UC (P<0.01). By contrast, there were no significant differences in the expression of miR-34a (Fig. 5B) and miR-106b (Fig. 5C) among the three groups.

Considering the target gene predicting results, the expression profiling and whether the candidate genes were previously reported to be involved in CRC or UC, three target genes of miR-31 [special AT-rich DNA-binding protein 2 (SATB2), zinc finger CCCH type-containing 12 (ZC3H12C) and Ras p21 protein activator 1 (RASA1)] were selected for validation. The RT-qPCR results revealed that the expression of SATB2 was significantly different among the UC, UC-LGD and UC-HGD samples (P<0.0001; Fig. 6A). The expression of SATB2 in patients with UC-LGD was significantly decreased compared with that in patients with UC (P<0.05). A more significant difference was observed between the UC-HGD and UC groups (P<0.0001). Contrary to the expression profile in the dataset GSE37283, there was no significant difference in the expression of ZC3H12C (Fig. 6B) and RASA1 (Fig. 6C) among the UC, UC-LGD and UC-HGD groups. In addition, the expression of SATB2 was indicated to be negatively correlated with that of miR-31 in all patients with UC (r=-0.5123, P=0.0001; Fig. 7).

miR-31 overexpression promotes CRC cell proliferation via repressing SATB2 expression in vitro. To confirm whether miR-31 promotes carcinogenesis via downregulating SATB2, CRC cells were transfected with miR-31 mimics and negative controls. Subsequently, RT-qPCR and cell proliferation assays were performed. miR-31 expression was confirmed to be increased in SW480 cells following transfection (P<0.0001; Fig. 8A). In parallel, the expression of SATB2 was downregulated in SW480 cells transfected with miR-31 mimics (P<0.01; Fig. 8B). Furthermore, overexpression of miR-31 promoted
SONG et al: miR-31 PROMOTES UCAN VIA DOWNREGULATING SATB2

SW480 cell proliferation, as indicated by the CCK-8 assay (P<0.01; Fig. 8C).

Discussion

In the present study, DEMs and DEGs were screened from expression profiles from the GEO database. The result of the bioinformatics analysis demonstrated that the mRNA and the miRNA expression data were evenly distributed. A visual presentation of the results of the enrichment analysis was also performed. Although the final candidate functional genes were not included in these signaling pathways, valuable information was obtained for future studies.

With a longer duration of UC, malignant transformation is the most serious complication (2). Histologically, the UCAN lesions may include LGD, HGD and invasive carcinoma (3). However, the pathogenesis of UCAN differs from that of sporadic CRC. Inflammation-associated microsatellite alterations were detected in the affected tissues of patients with UC, which indicated the presence of genomic mutations in the neoplastic tissue (23). The frequency of inflammation-associated microsatellite alterations was increased during the progression of UCAN (23). Glycosyltransferase ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 1 (ST6GALNAC1) was induced by M2-like macrophages, and ST6GALNAC1 alters the glycosylation status of the oncoprotein mucin 1, thereby promoting cancer development and progression in UC (24). However, the exact mechanisms underlying the pathogenesis of UCAN remain elusive.

miRNAs participate in a variety of biological processes in a large number of diseases via regulating their target genes. Indeed, numerous studies have proven the regulatory function of miRNAs in UC. Cytokines serve critical roles in the inflammatory process of UC and miR-124 was indicated to promote the inflammatory process by upregulating the expression of STAT3 (6). miR-31 and miR-155 may inhibit the expression of IL-13 receptor α1 (IL13RA1), thus regulating the IL-13 signaling pathway (7). Furthermore, miR-155 exerted an important effect on the NF-κB signaling pathway via targeting forkhead box O3a (25).

miR-31 has been implicated in a variety of diseases. As mentioned above, miR-31 was suggested to be overexpressed in the inflamed colonic mucosa in UC and it regulated IL-13 signaling by targeting IL13RA1 (7). miR-31 regulated not only cytokine receptor expression, but also the Hippo and Wnt signaling pathways, which promoted the generation of epithelium in mice with colitis (22). In addition to UC, miR-31...
was also upregulated in tissues from patients with Crohn's disease (26). However, the regulatory role of miR-31 is not limited to the progression of inflammation in UC. A number of studies have attempted to elucidate the regulatory function of miR-31 in the pathogenesis of colon cancer. Wang et al. (27) indicated that miR-31 expression was associated with CRC invasion and metastasis (39,40). Mechanistically, SATB2 expression was also indicated to be negatively associated with microsatellite instability that may impair 5-FU sensitivity in CRC (41). Furthermore, low SATB2 expression was determined to be common and to serve as a biomarker of UC-associated colorectal dysplasia (42). These results indicate that SATB2 may be involved not only in the progression of CRC, but also in the carcinogenesis of UCAN. The expression of SATB2 is regulated by multiple factors (43,44). It has been proven that miR-31 overexpression may repress SATB2 expression, resulting in CRC progression (43). Therefore, in the present study, SATB2 was selected as the target gene of miR-31 to be validated. It was observed that the expression of SATB2 decreased in a stepwise manner from inflamed epithelium to high-grade dysplasia and was negatively correlated with the expression of miR-31. This indicated that miR-31 promotes carcinogenesis in UCAN via downregulating SATB2. However, the downstream mechanism of SATB2 in the carcinogenesis of UCAN remains elusive. SATB1 is another member of the SATB protein family, which shares a high degree of sequence homology with SATB2. It has been reported that the dynamic balance between these two proteins regulates tumor invasion and metastasis (32). SATB1 acts as a positive regulator during these processes, whereas SATB2 serves the opposite role. SATB1 reportedly promotes tumor progression and metastasis through Wnt/β-catenin (45), retinoblastoma protein-E2 transcription factor signaling (46) and epithelial-to-mesenchymal transition-associated transcription factors (47). These regulating functions of SATB1 mentioned above may provide a starting point to investigate the downstream regulatory mechanism of SATB2 in the future, as further research on the downstream mechanism of SATB2 in UC is required.

In conclusion, in the present study, the DEMs and target genes were screened with the profiles from the GEO database. The regulatory function of miR-31 on SATB2 was investigated and it was demonstrated that miR-31 promotes tumorigenesis through downregulating SATB2. This result may provide a theoretical basis to further elucidate the pathogenesis of UCAN. However, there were certain limitations to the present study. The microarray data were from the GEO database rather than our institution. The presence of selection bias cannot be excluded.
In addition, the difference in the expression of miR-31 was not particularly notable, which may be attributed to inter-individual differences, although it was determined to be statistically significant. Further studies are required to confirm the present results and to further elucidate the pathogenesis of UCAN.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors’ contributions

KJ and BMW conceived the study and interpreted the data. WTL and RL designed the study. YS performed the experiments, analyzed the data and wrote the manuscript. RL also generated the figures and tables. BMW approved the manuscript to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Informed consent was obtained from all of the patients. This study was approved by the Ethics Committee of Tianjin Medical University General Hospital (Tianjin, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


