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miR-101 suppresses colon cancer cell migration through the regulation of EZH2

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ABSTRACT

Background: colorectal cancer (CRC) is one of the most prevalent types of malignancies worldwide. The incidence of CRC is steadily increasing due to extended life expectancy and aging-related genetic and epigenetic abnormalities. Dysregulation of microRNAs (miRNAs) has been implicated in CRC development.

Methods: the current study is a basic research study aimed at understanding the molecular mechanism of miR-101 in the pathogenesis of CRC using human samples in vivo and CRC cell lines in vitro. The miRNAs profile from human samples was analyzed by miRNA microarrays and the expression level of single miRNAs were confirmed by qRT-PCR. The validation of the direct target of miR-101 was performed by western blot assay. The cell mobility of CRC was assessed using the Transwell migration assay.

Results: downregulation of miR-101 was identified in 39 human CRC tissues and CRC cell lines (HT29 and SW620) when compared to their counterpart control. We further confirmed that the enhancer of zeste homolog 2 (EZH2), a histone methyltransferase, is a direct target of miR-101. Overexpression of EZH2 promoted CRC cell line migration and this effect was inhibited by forcing the expression of miR-
Thus, we conclude that miR-101 regulated colon cancer cell migration occurs at least partially, though targeting EZH2.

**Conclusion:** our study suggests that miR-101 functions as a tumor suppressor in CRC, and miR-101 may be a potential therapeutic target for CRC treatment.

**Keywords:** Colorectal cancer. miR-101. EZH2. Tumor suppressor. Cell migration.

**INTRODUCTION**

The incidence of colorectal cancer (CRC) and cancer-related disease has increased dramatically in the past 40 years and CRC has now become one of the most predominant cancers worldwide (1-3). CRC is ranked as the third most common type of cancer globally and approximately 10% of cancer-related mortality is related to CRC in Western countries. It is believed that both genetic and environmental factors contribute to the development of CRC (4-6). For example, most of the patients with gene mutations in DNA mismatch-repair genes or the adenomatous polyposis coli (APC) gene are more likely to develop CRC. A range of unhealthy lifestyle factors including heavy smoking, alcohol abuse and hyperphagia also correlated with an increased risk of colorectal cancer (2,7). A better understanding of the mechanism of CRC development may provide novel therapeutic approaches to prevent and treat CRC.

MicroRNAs (miRNAs) are small noncoding single-stranded RNAs comprising 18-25 nucleotides. The mature miRNA can bind with the 3’ untranslated region (UTR) of the target mRNA, leading to functional suppression of target mRNA through translational repression or degradation of target mRNA (8,9). Thus, miRNAs are recognized as key players in various cell functions (e.g., cell differentiation, proliferation, migration, and apoptosis), as well as in numerous pathological processes, including cancer initiation and progression (10). MiR-143 and miR-145 were the first miRNAs found to be downregulated in CRC and their functional roles have been extensively examined. Multiple genes have been confirmed as direct targets of miR-143 or miR-145 (11-13). For example, proto-oncogene tyrosine-protein kinase Yes (YES) and signal transducer and activator of transcription 1 (STAT1) have previously been identified as targets of...
miR-145, and their expression is associated with the development of CRC (14). Zhang et al. suggested that miR-143 impaired colon cancer cell proliferation and invasion through the regulation of metastasis-associated in colon cancer-1 (MACC1) (15). MiR-101 is another downregulated miRNA identified in CRC (16,17). However, the biological functions of miR-101 in the development and progression of CRC remain elusive.

In this study, we aimed to study the expression profile of miR-101 in colon cancer tissues and to address the functional target of miR-101 that may be associated with colon cancer cell migration.

MATERIAL AND METHODS

Patients and tissues samples

The current study is a basic research study. A total of 39 paired human colon carcinoma tissues with matched non-cancerous adjacent tissues were obtained from the Huaibei Normal University. The CRC samples and non-cancerous tissues were examined by two pathologists. All participating CRC patients were given written informed consent and the study protocol was approved by the Ethics Committee of the Huaibei Normal University. Participating subjects provided written informed consent.

Cell culture, reagents and antibodies

Human colon cancer cell lines (HT-29 and SW620) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in DMEM (Dulbecco’s Modified Eagle’s Medium; Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin and streptomycin (Gibco) at 37 °C in an incubator with 5% CO₂. Antibodies against EZH2 and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). The commercially available off-the-shelf miRNA-negative control (NC), miR-101 mimic precursor and miR-101 inhibitor, as well as a siRNA- negative control (NC) and si-EZH2 were purchased from Thermo Fisher Scientific (Waltham, MA, USA).
Microarray hybridization
A microarray was used and each probe was repeated three times on the microarray. MiRNAs were labeled with Hy5TM or Hy3TM fluorescent groups using the miRCURYTM Array Power Labeling reagent kit to form fluorescent probes. The background was removed from the signal value and scale normalization was performed. The ratio between groups > 2 times or < 0.5 times and a p value < 0.05 of the t-test indicated the miRNAs that were differentially expressed. A ratio > 2 times was defined as up-regulation and a ratio < 0.5 times was defined as down-regulation.

Western blot assay
Cells were collected and lysed in radioimmunoprecipitation buffer and homogenized on ice. The cell lysate was centrifuged at 13,000 g 4 ºC for 15 min and the supernatant was collected. The protein concentration was determined and 30 µg of total protein from each sample was loaded on an 8 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. After electrophoretic separation, the protein on the gel was transferred to a nitrocellulose membrane (Whatman, Germany). The membrane was blocked for 1 h and incubated with the specific primary antibodies overnight. The membrane was further probed with a secondary antibody for 1 h. The protein band was detected using enhanced chemiluminescence.

Quantitative RT-PCR (RT-qPCR)
The expression levels of miRNAs were determined using the TaqMan MicroRNA Assay Kit (Thermo Fisher Scientific) with primers specific for hsa-miR-101, hsa-miR-223, hsa-miR-605, hsa-miR-576-5p, miR-609 and hsa-miR-1253. The expression levels of RNU6B were used as an endogenous control for normalization. The comparative cycle threshold method (2^(-ΔΔCT)) was applied to determine the relative fold change in expression of the target gene transcript.

Cell migration assay
CRC cells were seeded in the Transwell chamber (8-µm pore size) purchased from Corning (Corning, NY, USA) and incubated in serum-free medium for 24 h. Then the
lower Transwell chamber was filled with DMEM mixed with 10% FBS as a chemoattractant. After 24 h, the Transwell chamber in each group was washed with phosphate-buffered saline buffer and fixed in cold alcohol and then stained with 0.5% crystal violet. The number of migrated cells was counted using light microscopy.

**Statistical analysis**

The results were analyzed using the SPSS 15 statistical software (IBM, Armonk, New York, USA). All data except miRNA microarray data were shown as the mean ± SD of three replicates. Differences between two groups were analyzed by the Student’s t-test and differences among multiple groups were analyzed by a one-way ANOVA analysis with a Tukey’s post hoc. The differences were recognized as significant at p < 0.05.

**RESULTS**

**miR-101 was downregulated in human colon cancer tissues**

To determine the miRNAs expression profile in colon cancer tissues and healthy normal tissues, a miRNA microarray assay was performed to profile the expression levels of mature miRNAs in four pairs of colon cancer tissues and matched adjacent healthy tissues. The heat map data from the miRNA microarray assay revealed six significantly down- and up-regulated miRNAs in adjacent normal tissues compared with those in colon cancer tissues (Fig. 1A). The down-regulation of miRNAs (miR-101, miR-609, and miR-1253) and up-regulation of miRNAs (miR-223, miR-605, and miR-576-5p) were further confirmed by Taqman RT-qPCR (Fig. 1B). Interestingly, we identified that miR-101 was the most significantly down-regulated miRNA in colon cancer tissues compared to normal tissues. Thus, we focused on investigating the expression profile and biological functional role of miR-101 in human colon cancer.

**miR-101 downregulation and EZH2 upregulation in colon cancer tissues**

To further validate our finding of down-regulation of miR-101 in colon cancer tissues, another 39 paired human CRC tissues and adjacent healthy normal tissues were
collected. The general characteristics of patients with colon cancer are shown in table 1. The expression levels of miR-101 in these samples were determined by Taqman RT-qPCR and the results demonstrated that the expression levels of miR-101 were indeed substantially decreased in CRC tissues compared with adjacent normal tissues (Fig. 1C). Interestingly, we also found that contrary to the results of miR-101 expression, the expression levels of EZH2 were evidently increased in colon cancer tissues compared to adjacent normal tissues (Fig. 1D).

**Modulation of miR-101 in colon cancer cell lines**

To study the functional role of miR-101 in human colon cancer cells, we aimed to modulate the expression levels of miR-101 in CRC cells. To reach this goal, HT29 and SW620 were transfected with a miRNA-negative control (NC), miR-101 mimic precursor and miR-101 inhibitor. Taqman RT-qPCR results showed that the expression levels of miR-101 were significantly enhanced or decreased after transfection with the miR-101 mimic precursor or miR-101 inhibitor, respectively (Fig. 1E and F).

**EZH2 is a direct target of miR-101**

EZH2, a histone methyltransferase, functions to initiate epigenetic silencing of cell death genes. It was reported to be a direct target of miR-101 (18) as the 3’ untranslated region of EZH2 mRNA contains the “seed region” of miR-101 (Fig. 2A). To validate this conclusion, HT29 and SW620 were transfected with the miRNA-negative control (NC), miR-101 mimic precursor, miR-101 inhibitor, a siRNA- negative control (NC) and si-EZH2 as a positive control. The western blot results showed that si-EZH2 effectively decreased EZH2 protein levels and overexpression of miR-101 decreased, whereas suppression of miR-101 enhanced the expression levels of EZH2 in HT29 and SW620 (Fig. 2B and C). Collectively, these results suggested that EZH2 is a direct target of miR-101.

**miR-101 regulated colon cancer cell migration through targeting EZH2**

A Transwell migration assay was used to investigate the effect of miR-101 on the
migration of colon cancer cells. HT29 and SW620 were transfected with the miRNA-negative control (NC), miR-101 mimic precursor and miR-101 inhibitor as well as siRNA-negative control (NC) and si-EZH2. The cell migration results showed that knockdown of EZH2 levels via miR-101 or si-EZH2 overexpression significantly impaired the migration ability of colon cancer cells. On the contrary, increasing EZH2 levels via miR-101 inhibition dramatically promoted the migration ability of colon cancer cells (Fig. 3A-C). These results suggested that miR-101 may regulate the migration of colon cancer cells by targeting EZH2.

DISCUSSION

In this study, we identified using miRNA microarray technology that miR-101 was the most significantly down-regulated miRNAs in colon cancer tissues compared to normal tissues. We further confirmed our finding in 39 paired human colon cancer tissues and adjacent normal tissues as well as two colon cancer cell lines (HT29 and SW620) and one normal colon epithelial cell line. Many miRNAs are expressed in a tissue and cell type specific manner. For example, miR-31 was reported to be downregulated in breast, liver, ovarian and brain cancer (19-22). However, upregulation of miR-31 was found in lung, colon and cervical cancer (23-25). Interestingly, miR-101 was reported to be down-regulated in almost all human carcinoma tissues, which strongly suggests that miR-101 functions as a universal tumor suppressor in diverse malignant tumors (26). Consistent with earlier research, we also confirmed that miR-101 is substantially decreased in CRC tissues, suggesting that miR-101 may also function as a tumor suppressor in CRC development.

It is well established that one miRNA could regulate the expression of various target genes, while one gene could be targeted by different miRNAs. This added the difficulty of identifying the specific target(s) of miR-101 in different cancer types. For example, miR-101 was reported to target sphingosine kinase 1 (SphK1) and resulted in the suppression of tumor angiogenesis in colon cancer (27). miR-101 was also shown to regulate the hypoxia inducible factor-1α (HIF-1α) through targeting von Hippel-Lindau (VHL), a negative regulator of HIF-1α. Over-expression of miR-101 decreased VHL levels and subsequently stabilized HIF1α, leading to enhanced HIF1α-
dependent cell apoptosis in breast cancer (28). Importantly, EZH2 was reported as a target of miR-101 in multiple human cancers, including non-small cell lung cancer, epithelial ovarian cancer, gastric cancer and osteosarcoma (29-33). It was shown that miR-101 inhibited cancer cell proliferation via directly decreasing EZH2 expression in lung cancer (29). Furthermore, miR-101 decreased the expression of EZH2, which mediated the transcriptional repression of p21, thus leading to the inhibition of cell metastasis (34). Similarly, Jiang et al. demonstrated that miR-101 plays a key role in GlcNAc and EZH2 regulatory signaling in the mediation of CRC metastasis (35). In addition, miR-101 was shown to reduce EZH2 levels and enhance the E-cadherin levels in gastric cancer (33). However, the question of whether EZH2 is a direct target of miR-101 in colon cancer has never been addressed by previous studies. Here, we demonstrated that EZH2 is indeed a direct target of miR-101 in colon cancer cells. We further elucidated that down-regulation of EZH2 via miR-101 or si-EZH2 overexpression decreased the migration ability of colon cancer cells. However, more studies are needed to clarify whether EZH2 is the main factor that modulates the migration of colon cancer cells. Taken together, we found that downregulation of miR-101 leads to upregulation of EZH2 in human CRC. These results suggest that miR-101 may regulate the migration of colon cancer cells through targeting EZH2, indicating that miR-101 might provide a novel and effective insight in the molecular targeting treatment for colon cancer patients.

FUNDING
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REFERENCES

Accepted Article


10. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol 2019;20:21-37. DOI: 10.1038/s41580-018-0045-7


STAT1 in colon cancer cells. PLoS One 2010;5:e8836. DOI: 10.1371/journal.pone.0008836


and functions as an oncomir in cervical cancer via targeting ARID1A. Gynecol Oncol 2014;134:129-37. DOI: 10.1016/j.ygyno.2014.04.047


Table 1. General characteristics of patients with colon cancer

<table>
<thead>
<tr>
<th></th>
<th>Colon cancer patients (n = 39)</th>
</tr>
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<tbody>
<tr>
<td>Age, mean (SD)</td>
<td>64.36 (6.97)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>20</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
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<tr>
<td>Tumor size (cm)</td>
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<tr>
<td>d &gt; 4</td>
<td>18</td>
</tr>
<tr>
<td>d ≤ 4</td>
<td>21</td>
</tr>
<tr>
<td>Degree of differentiation</td>
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<td>High and medium</td>
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<tr>
<td>Low</td>
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<td>Cancer stage</td>
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<td>I-II</td>
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<tr>
<td>III-IV</td>
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<td>Family history with colon cancer</td>
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</tr>
<tr>
<td>No</td>
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</table>
Fig. 1. miR-101 expression and manipulation in colon cancer. A. miRNA microarray of tissue samples from four colon cancer patients. Heat map data analysis of the differentially expressed miRNA in four pairs of colon cancer tissues and adjacent normal tissues by miRNA microarray assays. B. Relative fold change of six miRNAs in colon cancer and adjacent normal tissues measured by a microarray. miR-101 is down-regulated in colon cancer tissues. The expression levels of miR-101 (C) and EZH2 mRNA (D) in colon cancer and adjacent normal tissues were analyzed by qRT-PCR, n = 39 for each group. Manipulation of miR-101 expression in colon cancer cell lines. E and F. HT29 (E) and SW620 (F) cells were transfected with miR-101 mimic precursor, miRNA-negative control (NC) and miR-101 inhibitor. The expression levels of miR-101 were detected by qRT-PCR. Data are shown as the mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001.
Fig. 2. EZH2 is a direct target of miR-101. A. The predicted sites of miR-101 binding to the 3'-UTR region of EZH2 are shown. B and C. HT29 (B) and SW620 (C) were transfected with miR-101 mimic precursor, miRNA-negative control (NC) and miR-101 inhibitor as well as with a siRNA-negative control (NC) and si-EZH2. The protein levels of EZH2 were detected by western blot and quantified. Data are shown as the mean ± SD of three replicates. *p < 0.05; **p < 0.01.
Fig. 3. miR-101 suppresses the migration of HT29 and SW620 cell lines. (A) Images from Transwell migration assays of HT29 and SW620 cells transfected with the indicated mimics or inhibitors. B and C. The migrated HT29 (B) and SW620 (C) cells were analyzed. Data are shown as the mean ± SD of three replicates. *p < 0.05; **p < 0.01.