**Abstract.** – OBJECTIVE: Long noncoding RNAs (lncRNAs) have been identified in various malignant tumors and determined to play an essential role in terms of cancer progression. In this study, we aimed at exploring the molecular mechanism of LINC00963 in colorectal cancer (CRC).

PATIENTS AND METHODS: The mRNA expressions of LINC00963, miR-124-3p and FZD4 in CRC tissues and cells were detected by qRT-PCR. CCK-8 and transwell assay were chosen to measure the CRC cell vitality. Western blot analysis was performed to assess the expression level of FZD4 in CRC. The correlation between LINC00963 and miR-124-3p or miR-124-3p and FZD4 was appraised by Dual-Luciferase reporter assay.

RESULTS: In this study, LINC00963 was significantly upregulated in CRC tissues and cells. Functionally, LINC00963 knockdown inhibited cell progression in CRC. The results verified that LINC00963 can restrain the expression of miR-124-3p. Moreover, FZD4 restored the inhibitory effect of miR-124-3p on the progression of CRC cells. Besides that, we preliminarily verified that FZD4 was a direct target gene of LINC00963/ miR-124-3p axis in CRC.

CONCLUSIONS: Our study demonstrated that LINC00963/miR-124-3p/FZD4 played a curial role in cell proliferation and migration in CRC. In addition, LINC00963 can be a possible therapeutic and diagnostic target for CRC treatment.

**Key Words:**
Colorectal cancer, LINC00963, MiR-124-3p, FZD4.

**Introduction**

Colorectal cancer (CRC) is one of the most prevalent malignant tumors in the world. Nearly 80% CRC patients are diagnosed in late stage, since the early symptoms of CRC are relatively insidious. Wu et al. have shown that the clinical incidence and mortality of CRC are on the rise in China year by year. Around 1000 people in China are diagnosed with CRC every day, and one patient dies from CRC every three minutes. However, the mechanism of its initiation and progression is not yet entirely clear. Therefore, in-depth investigation of early diagnosis and targeted gene therapy have impartment values to effective treatment of CRC. With the development of oncomolecular biology, cytogenetics, immunology and other subjects, people have made great progress in tumor pathogenesis, prevention and treatment.

Long non-coding RNAs (lncRNAs) are a group of RNAs that not encoded proteins and be greater than 200 bp in length. Recently, researches have indicated that lncRNAs play important effects in apoptosis, proliferation, migration, invasion, etc., and their roles in the tumorigenesis and progression have also attracted increasing attention. Su et al. found that LINC01116 promoted migration and invasion in gastric cancer by positively regulating CASC11. LncRNA ENST00000434223 was found to inhibit the proliferation and metastasis by suppressing the Wnt/β-catenin signal pathway in renal cancer. LINC00963 is located on chromosome 9 with 25027 bp in length and has been verified to be differentially expressed and play a part in tumorigenesis of various human tumors, such as in osteosarcoma, melanoma, prostate cancer and renal cancer. However, the molecular mechanism and function of LINC00963 in CRC are still not clarified.

In the present research, we will investigate the function of LINC00963 on the tumorigenesis and development of CRC. Next, the mechanism of LINC00963/miR-124-3p/FZD4 in CRC was also expounded.
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Patients and Methods

Patient Tissue Samples
84 CRC tissues and paracancer tissues were obtained from the Tianjin People’s Hospital (Tianjin, China). These specimens were put into liquid nitrogen, then stored at -80°C for the following experiments. The samples were evaluated according to the classification criteria of World Health Organization. The CRC progression was classified by the CRC guidelines outlined in the seventh edition of the American Joint Committee on Cancer’s staging manual. All enrolled CRC patients did not receive preoperative radiotherapy, chemotherapy, radiofrequency ablation or other adjuvant therapy. Before the experiment, the signed written informed consents were procured from all patients. We started the research with the approval of the Ethics Committee of Tianjin People’s Hospital.

Cell Culture and Cell Lines
In this study, CRC cell lines LOVO and SW620 were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). HCT116, SW1990 and normal CRC cell line NCM460 were purchased from BeNa Culture Collection (Beijing, China). CRC cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium containing 10% fetal bovine serum (FBS) at 37°C and 5% CO₂.

Real-Time Quantitative PCR
All RNA samples were extracted from CRC tissues and cells by TRizol reagent (Invitrogen, Carlsbad, CA, USA). Then, RNAs were reverse-transcribed into complementary according to PrimeScript RT reagent kit (Invitrogen, Shanghai, China). Bio-Rad CFX96 and SYBR Green Premix Taq II (TaKaRa, Dalian, China) were used for qRT-PCR. Finally, the target RNA relative expression level was quantified by LightCycler 96 thermocycle (Roche, Basel, Switzerland). LINC00963 and miR-124-3p expression were normalized to GAPDH, while FZD4 was standardized to β-actin. All primer sequences are showed in Table I.

Cell Counting Kit-8 (CCK-8) Assay
CRC cells (2×10⁴ cells/well) were cultured in 96-well plates at 37°C, 5% CO₂. After incubation, CCK-8 reagents (Dojindo Molecular Technologies Inc., Kyushu, Japan) were added to each well in accordance with protocol at 24, 48, 72 and 96 h. Accordingly, we measure the relative optical density (OD) values at 450 nm.

Transwell Assay
Transwell chambers (8 μm; Millipore, Billerica, MA, USA) were performed to assess the migration ability of CRC cells. First, 1 × 10⁵ cells were inoculated in the upper transwell compartment. Then, 10% FBS was added into the lower compartment. Cells were fixed and stained with 0.5% crystal violet, after cultivated at 37°C for 24 h. Finally, counted the migrating cells numbers under an optical microscope (Olympus Corp., Tokyo, Japan).

Dual-Luciferase Activity Assay
LINC00963-mut or LINC00963-wt, miR-NC or miR-124-3p mimics and FZD4-mut or FZD4-wt were co-transfected into CRC cells by using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). SW620 and HCT116 cells were cultured in the incubator at 37°C, 5% CO₂, and 95% humidity. After 48 hours, Dual-Luciferase reporter assay kit was used to assess the Luciferase activity.

Table I. Primer sequences in qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>LINC00963</td>
<td>Forward 5'-GTCAGGCCACTCTGCTACTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCACTGCGATGGTTGTGTC-3'</td>
</tr>
<tr>
<td>miR-124-3p</td>
<td>Forward 5'-ACGGGATCCTCTTATTCCATCTTCTACCC 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CGGAATTCCTGGCTCGGTGCTG TCC 3'</td>
</tr>
<tr>
<td>FZD4</td>
<td>Forward 5'-CAGTGAGGCATGGAGGTGTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AAAGAGCTCAAGGGGCCATC-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward 5'-AACAGGCTCAAGGGGCCATC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CCACTGCGATGGTTGTGTC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-CCTGACCTGAGGATGGTGCAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCTGTGATGGGAGGTGTC-3'</td>
</tr>
</tbody>
</table>
Western Blot Assay
Protein samples were isolated by the radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific, Waltham, MA, USA). Total proteins were isolated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. After incubating with 5% non-fat milk, membranes were incubated with primary antibody (ab16901, 1:1000, Abcam, MA, USA) at 4°C overnight. Then, the membranes were incubated with horseradish peroxidase (HRP) labelled secondary antibody (ab205719, 1:2000, Abcam, MA, USA) at 37°C for another 1 hour. Finally, the protein bands were revealed by enhanced chemiluminescence (ECL; Thermo Fisher Scientific Inc., Waltham, MA, USA).

Statistical Analysis
Data were analyzed by SPSS 19.0 (IBM, Armonk, NY, USA) and presented as mean ± SD from at least three biological replicates. Tukey’s post-hoc test of One-way ANOVA was used to analyze the multiple comparisons. Student’s t-test was performed to analyze the differences between two groups. Moreover, the correlation analysis was analyzed by linear regression. Differences were defined as significant with \( p < 0.05 \).

Results

LINC00963 Was Upregulated in CRC Tissues and Cells
To investigate the expression of LINC00963 in CRC, qRT-PCR analysis was performed in 84 CRC tissues. As Figure 1A presented, LINC00963 expression was notably higher in CRC tissues than in normal tissues \((p<0.01)\). We inspected the expression of LINC00963 in four human CRC cell lines (SW1990, LOVO, SW620 and HCT116) and normal colorectal cell line (NCM460). The expression level of LINC00963 in CRC cell lines was significantly higher than that in normal cell line NCM460 \((p<0.01\); Figure 1B). The correlation between LINC00963 and clinical characteristics of CRC patients was listed in Table II. LINC00963 expression was found to be closely related with TNM stage \((p=0.005)\) and tumor size \((p=0.031)\). Furthermore, we classified 84 CRC patients into the low expression group and high expression group based on the mean value of LINC00963 expression. The Kaplan Meier survival analysis revealed that patients with high expression level of LINC00963 had remarkably shorter survival time than the low group (Figure 1C). Our results indicated that LINC009663 was involved in CRC.

LINC00963 Silencing Suppressed the Progression of CRC Cells
To investigate the biological function of LINC00963 in CRC progression, we reduced the expression of LINC00963 in SW620 and HCT116 cells by transfection of si-LINC00963 (Figure 2A). Thereafter, CCK-8 assay was used to verify CRC cell proliferation. The results revealed that LINC00963 knockdown suppressed the proliferation of SW620 and HCT116 cells \((p<0.05; p<0.01\); Figure 2B). Additionally, transwell assay displayed that si-LINC00963 inhibited the migration when transfected into SW620 and HCT116 cells \((p<0.01\); Figure 2C). Therefore, our results verified that LINC00963 silencing restrained CRC cell proliferation and migration.
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LINC00963 Can Act as a Sponge of MiR-124-3p

The potential target genes of lncRNA were searched in the StarBase database which displayed that LINC00963 contained the conserved target sites of miR-124-3p (Figure 3A). Next, the results indicated that the Luciferase activity of LINC00963-wt was reduced by miR-124-3p mimics transduction. However, the Luciferase activity of LINC00963-mut remains unchanged by miR-124-3p mimics ($p<0.01$; Figure 3B). Subsequently, qRT-PCR analysis revealed that LINC00963 knockdown upregulated miR-124-3p expression significantly, while LINC00963 vector reduced its expression ($p<0.01$; Figure 3C). As showed in Figure 2. LINC00963 silencing restrained the proliferation and migration of CRC cells.

Table II. Correlation between the expression level of LINC00963 and clinical characteristics of CRC patients ($n=84$).

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>No. of cases $n=84$</th>
<th>LINC00963 expression</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low ($n=35$)</td>
<td>High ($n=49$)</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td>0.488</td>
</tr>
<tr>
<td>≤60</td>
<td>30</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>&gt;60</td>
<td>54</td>
<td>21</td>
<td>33</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td>0.793</td>
</tr>
<tr>
<td>Male</td>
<td>49</td>
<td>21</td>
<td>28</td>
</tr>
<tr>
<td>Female</td>
<td>35</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td>0.031*</td>
</tr>
<tr>
<td>≤5 cm</td>
<td>51</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>&gt;5 cm</td>
<td>33</td>
<td>9</td>
<td>24</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td>0.005**</td>
</tr>
<tr>
<td>I-II</td>
<td>40</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>III-IV</td>
<td>44</td>
<td>12</td>
<td>32</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>0.128</td>
</tr>
<tr>
<td>Absent</td>
<td>47</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

*$p<0.05$, the difference is significant. **$p<0.01$, the difference is highly significant.
3D, miR-124-3p was lower expressed in CRC tissues than normal tissues \((p<0.01)\). Furthermore, Pearson’s correlation analysis displayed that miR-124-3p and LINC00963 expression presented a negative correlation in 84 CRC tissues \((R^2=0.6318, p<0.001; \text{Figure 3E})\). Our results suggested that miR-124-3p can directly target LINC00963 in CRC.

**LINC00963 Modulated CRC Progression Via MiR-124-3p**

Firstly, the expression of miR-124-3p in CRC cells was verified to be remarkably decreased compared with the normal CRC cells \((p<0.001; \text{Figure 4A})\). To investigate the function of LINC00963/miR-124-3p in the progression of CRC, miR-124-3p mimics or HEIH vector was transfected into SW620 and HCT116 cells \((p<0.01; \text{Figure 4B})\). CCK-8 assay revealed that the decrease of cell proliferation stimulated by miR-124-3p mimics was mitigated by LINC00963 vector \((p<0.01; \text{Figure 4C})\). Additionally, transwell assay indicated that LINC00963 vector also alleviated the reduction of cell migration ability stimulated by miR-124-3p overexpression in SW620 and HCT116 cells \((p<0.01; \text{Figure 4D})\). In conclusion, these results indicated that LINC00963 promoted cell progression in CRC via sponging miR-124-3p.

**FZD4 Was a Direct Target of MiR-124-3p**

TargetScan software predicted that miR-124-3p had binding sites with FZD4 (Figure 5A). As shown in Figure 5B, FZD4 was upregulated in 84 CRC tissues \((p<0.01; \text{Figure 5B})\). Next, upregulation of FZD4 was detected in four CRC cell lines compared with normal CRC cell line \((p<0.01; \text{Figure 5C})\). Subsequently, miR-124-3p mimics remarkably descended the Luciferase activity of FZD4-wt, but not FZD4-mut, in SW620 and HCT116 cells \((p<0.01; \text{Figure 5D})\). Furthermore, miR-124-3p overexpression significantly reduced the FZD4 expression, and miR-124-3p inhibitor raised the expression of FZD4 in CRC cells \((p<0.01; \text{Figure 5E})\). Additionally, miR-124-3p has a negative correlation compared with FZD4 expression levels in CRC tissues \((R^2=0.6165, p<0.001; \text{Figure 5F})\). Inversely, there was a positive correlation between LINC00963 and FZD4 \((R^2=0.6307, p<0.001; \text{Figure 5G})\). To conclude, our results suggested that FZD4 was a direct target gene of miR-124-3p.

**LINC00963 Modulated CRC Progression Via MiR-124-3p/FZD4**

To detect whether LINC00963 regulated FZD4 expression via miR-124-3p, LINC00963 vector or miR-124-3p inhibitor was transfected into SW620 and HCT cells with si-FZD4 \((p<0.001; \text{Figure 6A})\).
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6A). CCK-8 and transwell assay discovered that the decrease of cell proliferation and migration induced by si-FZD4 was mitigated by LINC00963 vector or miR-124-3p inhibitor (p<0.001; Figure 6B-C). These data verified that LINC00963 exerted an oncogenic function by regulating miR-124-3p/FZD4 in CRC.

**Discussion**

CRC is the most common malignant tumor of alimentary tract worldwide. Unfortunately, nearly half of CRC patients survive less than five years due to low early diagnosis rate\(^6\). Therefore, the development of molecular biomarkers is helpful
Figure 5. FZD4 was a direct target of miR-124-3p. A, MiR-124-3p was predicted to have binding sites with FZD4 by TargetScan. B, Expression of FZD4 in 84 CRC tissues. C, Expression of FZD4 in CRC cell lines. D, Interaction between miR-124-3p and FZD4 in SW620 and HCT116 cells was examined by Luciferase assay. E, Protein level of FZD4 was measured with miR-124-3p mimics or inhibitor transfection. F, FZD4 had a negative correlation with miR-124-3p in CRC tissues. G, FZD4 was positively correlated with LINC00963 in CRC tissues. **p<0.01
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Figure 6. LINC00963 regulated FZD4 via sponging miR-124-3p. A, FZD4 expression was measured with si-FZD4 transfection in LINC00963 vector or miR-124-3p inhibitor. B, Cell proliferation was measured with si-FZD4 transfection in LINC00963 vector or miR-124-3p inhibitor. C, Cell migration was measured with si-FZD4 transfection in LINC00963 vector or miR-124-3p inhibitor (scale bar=100 μm). **p<0.01

to early diagnose and reduce the mortality rates in CRC. Numerous reports have shown that lncRNAs are involved in a variety of tumors, such as breast cancer, renal cancer, gastric cancer, etc. Recently, lncRNA PCAT-1, lncRNA DLX6-AS1, LINC01234 and lncRNA AK001058 have been verified to play an important effect in CRC. LINC00963 is a novel lncRNA that plays a carcinogenic role in several human cancers. In hepatocellular carcinoma, LINC00963 was upregulated and significantly promoted the proliferative ability of HCC cells. Jiao et al verified that LINC00963 upregulation promoted melanoma cell growth and metastasis through regulating miR-608. However, the function of LINC00963 has not been explored in CRC by now. In this
study, we first verified that LINC00963 was aberrantly expressed in CRC tissues and CRC cells. Moreover, the upregulated expression of LINC00963 was found to be remarkably related to tumor size and TNM stage. Second, we performed functional experiments to detect the role of LINC00963 in CRC. The results displayed that knockdown of LINC00963 suppressed the CRC cell proliferation and migration.

Recently, lncRNAs have been reported to function as ceRNAs by targeting specific miRNAs in tumor progression. In osteosarcoma, LINC00963 promoted cell proliferation and invasion by regulating miR-204-3p. Wang et al. showed that LINC00963 regulated CSCC progression through miR-1193/SOX4 axis. In this research, miR-124-3p was predicted as a target of LINC00963 by the bioinformatics software. MiR-124-3p has been reported as a tumor inhibitor, and suppressed tumor metastasis in various cancers. Fu et al. discovered that miR-124-3p overexpression restrained proliferation and induced cell apoptosis in bladder cancer. Consist with the previous researches, we discovered that miR-124-3p was low expressed in CRC tissues and cell lines. Our results have shown that the decrease of cell progression stimulated by miR-124-3p mimics were mitigated by LINC00963 vector. The tumor inhibitory effect and expression level of miR-124-3p in CRC progression was consistent with the previous reports.

FZD4 (frizzled 4) is a transmembrane protein that crosses the membrane, which belongs to the frizzled receptor family. FAD4 protein is featured with a conserved cysteine-rich ligand binding region (CRD), and functions in Wnt signal transduction. Currently, FZD4 has been reported to be involved in the signaling process in a variety of malignant tumor cells and participates in cell proliferation, transdifferentiation and carcinogenesis. In our study, FZD4 was upregulated in CRC tissues and CRC cells. Similarly, upregulation of FZD4 was also detected in bladder cancer, and FZD4 overexpression restored the inhibiting effect of miR-101 on the invasion and migration of bladder cancer cells. Consistent with previous findings, we found that silencing of FZD4 reduced the CRC cell proliferation and migration, but upregulation of LINC00963 and miR-124-3p downregulation reversed the alteration. Consequently, these results suggested that LINC00963/miR-124-3p axis promoted CRC progression by upregulating FZD4.

**Conclusions**

For the first time we found that LINC00963 is upregulated in CRC, and overexpression of LINC00963 promotes CRC cell proliferation and migration through modulating miR-124-3p and upregulating FZD4 expression. Moreover, we revealed that LINC00963 is a promising biomarker target for CRC therapy.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

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