

Original Research

miR-532 promotes colorectal cancer invasion and metastasis by targeting NKD1

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Abstract: The aim of this study was to investigate the effect of microRNA-532 (miR-532) on invasion and metastasis of colorectal cancer (CRC) cells, and the underlying mechanism. Human CRC cell line (HCT116) and normal colon (FHC) cells were used for this study. The cells were transfected with naked cuticle homolog 1 (NKD1) overexpression plasmid, miR-532 mimics, miR-532 inhibitor or miR-532 non-homologous sequence using lipofectamine 2000. Real-time quantitative polymerase chain reaction (qRT-PCR) was used to determine the expression of miR-532 in CRC cells, and a combination of scratch and Transwell assays was used to assess the effect of miR-532 on migration and invasion of CRC cells. Western blotting was used to determine the effect of miR-532 on NKD1 expression in CRC cells. Bioinformatics analysis and luciferase reporter gene assay were used to assess the regulatory effect of miR-532 on NKD1. The expression of miR-532 was upregulated in CRC cells relative to normal colon cells ($p < 0.05$). The HCT116 cells transfected with miR-532 mimics migrated faster than those of miR-532 negative control (miR532-NC) group ($p < 0.05$). The migration ability of HCT116 cells transfected with miR-532 inhibitor was significantly reduced, when compared with that of miR532-NC group ($p < 0.05$). The invasive ability of HCT116 cells transfected with miR-532 mimics was significantly higher than that of miR532-NC cells ($p < 0.05$). However, inhibition of miR-532 expression significantly reduced the invasive ability of HCT116 cells ($p < 0.05$). Results of bioinformatics showed that miR-532 had specific binding sequence with the 3'UTR region of NKD1. After cloning the sequence into the luciferase reporter plasmid, miR-532 significantly inhibited the expression of NKD1 ($p < 0.05$). However, miR-532 had no inhibitory effect on mutated NKD1 3'UTR ($p > 0.05$). Results of Western blotting showed that increased miR-532 expression significantly reduced the expression of NKD1, while decreased miR-532 expression promoted the expression of NKD1 ($p < 0.05$). Overexpression of NKD1 significantly down-regulated miR-532 overexpression and promoted CRC cell invasion and metastasis ($p < 0.05$). miR-532 is highly expressed in CRC cells and directly inhibits NKD1 expression, while enhancing invasion and metastasis of CRC cells. It promotes the development of CRC by inhibiting the expression of NKD1.

Key words: Colorectal cancer; miR-532; invasion, migration; Expression.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors with high incidence of mortality in the world. Common treatments for CRC include resection, radiotherapy and chemotherapy (1). One of the mechanisms of development of CRC is the mutation of tumor suppressor genes. Studies have shown that CRC development and prognosis are linked to molecular changes in different genes (2). However, the mechanism driving CRC tumor transformation remains unclear. Therefore, CRC treatment requires elucidation of the molecular mechanism (s) that underlie the pathogenesis of CRC and as well as new molecular targets.

MicroRNAs (miRNAs) disrupt or inhibit translation of homologous mRNAs, thereby maintaining stability of the chromosome and regulating gene expression (3). Reports have shown that miRNAs also act as oncogenes or tumor suppressors, playing key roles in the development of cancer (4-6). MicroRNA-532 (miR-532) is a potential biomarker for deep vein thrombosis (7), and an early diagnostic marker for lung adenocarcinoma (miR-532, miR-628-3p and miR-425-3p) (8). The role of miR-532 in the pathogenesis of CRC and its regulatory mechanisms have not been fully elucidated. The

present study investigated the effect of miR-532 on invasion and metastasis of CRC cells, and the underlying mechanism.

Materials and Methods

Materials

Fetal bovine serum (10 %) was product of Sangon Co., Ltd. (China). Dulbecco's Modified Eagle/Ham's F12 (DMEM/F12) medium was obtained from Hyclone (USA). Lipofectamine 2000 was purchased from Invitrogen (USA); NKD1 and GAPDH genes were products of CST (USA), while luciferase reporter assay kit was purchased from Biyuntian Co., Ltd. (China). The qPCR primers were products of Shanghai Xingyuan Ruimin Biological Engineering Co., Ltd, and Trizol total RNA extraction kit was obtained from Shanghai Chaoyan Biotechnology Co., Ltd.

Cell culture and transfection

Human CRC cell line (HCT116) and normal colon (FHC) cells were cultured in 10 % fetal bovine serum (FBS) supplemented DMEM/F12 medium at 37 °C and 5 % CO₂ until the cells attained 80 % confluency. Cells in logarithmic growth phase were used for this study.

The cells (2×10^5 cells/well) were seeded into 6-well plates and incubated overnight. Thereafter, they were randomly assigned to four groups: NKD1 overexpression group, miR-532 overexpression group, miR-532 low expression group and miR532-NC group. The cells were transfected with NKD1 over-expression plasmid, miR-532 mimics, miR-532 inhibitor or miR-532 non-homologous sequence using lipofectamine 2000. After 24 h of transfection, the plasmid vector lipofectamine 2000 diluted in Opti-MEM medium was added and incubated for another 6 h, after which it was replaced with complete culture medium.

Quantitative RT-PCR

Trizol RNA extraction reagent was used to extract total RNA from cells of each group, while cDNA synthesis kit was used to perform the cDNA synthesis reaction according to the instructions of the manufacturer. Light Cycler 1536 Real-time PCR Detection System was used for the estimation of the expressions of mRNAs of miR-532 by quantitative RT-PCR. Variation in the cDNA content was normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR reaction system (20 μ l) consisted of 6.4 μ l of dH₂O, 1.6 μ l of gene-specific primer (10 μ M), 2 μ l of synthesized cDNA and 10 μ l of SYBR Premix Ex Taq™ II. The miR-532 upstream primer sequence was 5'CGCATGCCTTGAG-TGTAGGAC3'; downstream was 5'GTGCAGGTCC-GAGT3', and the Ct value of U6 was taken as the internal parameter. The relative expression level of miR-532 was calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blotting

The cells were washed with PBS and ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease inhibitor was used to lyse them. The resultant lysate was centrifuged at 12,000 rpm for 10 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (30 μ g) from each sample was separated on a 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min. Subsequently, non-fat milk powder (3 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 °C and incubated to block non-specific binding in the blot. Incubation of the blots with primary antibodies of NKD1 and GAPDH at a dilution of 1 to 500 was performed overnight at 4 °C. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1 h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using Bio-rad gel imaging system, and the respective protein expression levels were normalized to that of GAPDH which was used as a standard reference.

Scratch test

Cells in logarithmic growth phase were seeded into 6-well plates until they attained 80 % confluency, and scratches were made on cell monolayers. After washing thrice with serum-free medium, the cells were further cultured for 24 h, and then observed and photographed.

In vitro cell migration and invasion assay (Transwell invasion experiment)

The cells were placed in Transwell chamber coated with substrate and cultured in serum-free medium. A medium containing 10 % fetal bovine serum was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope.

Luciferase reporter gene assay

The NKD1 3'URT region and the mutated NKD1 3'URT region were cloned into the psiCHECK2 vector, and simultaneously the miR-532 mimics were transfected with colon cancer cells. After 48 h of transfection, luciferase activity was determined using luciferase reporter assay kit.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism (7.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

Results

Expression level of miR-532 in CRC cells

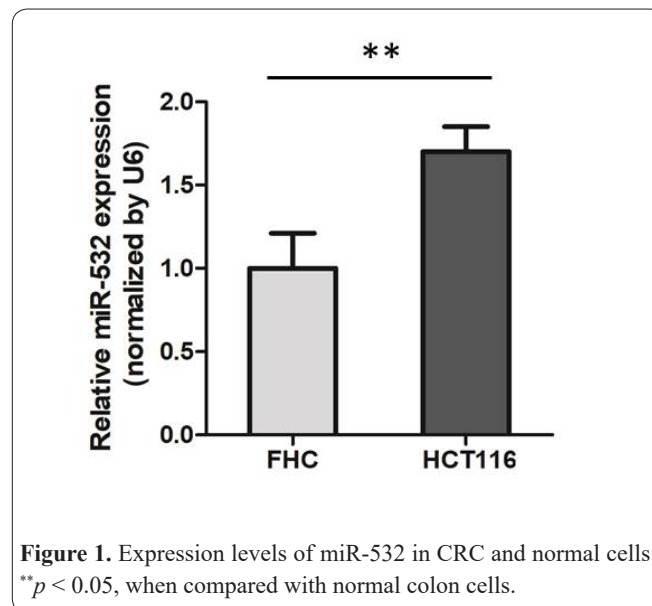
As shown in Figure 1, miR-532 expression was upregulated in CRC cells, relative to normal colon cells ($p < 0.05$).

Migration and invasion of CRC cells

The HCT116 cells transfected with miR-532 mimics migrated faster than those of miR532-NC group ($p < 0.05$). The migration ability of HCT116 cells transfected with miR-532 inhibitor was significantly reduced, when compared with that of miR532-NC group ($p < 0.05$; Figure 2).

Effect of miR-532 expression on invasion of CRC cells

The invasive ability of HCT116 cells transfected with miR-532 mimics was significantly higher than that



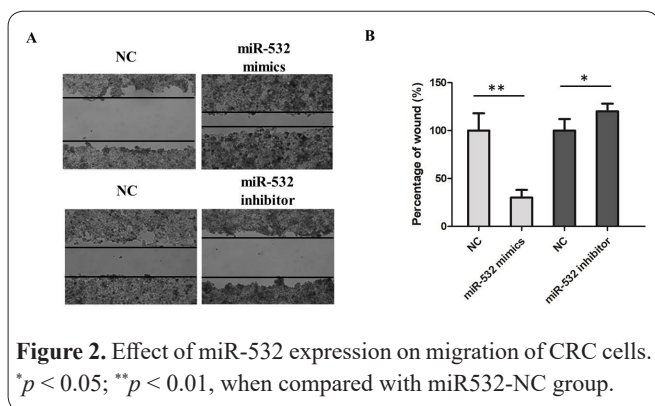


Figure 2. Effect of miR-532 expression on migration of CRC cells. * $p < 0.05$; ** $p < 0.01$, when compared with miR532-NC group.

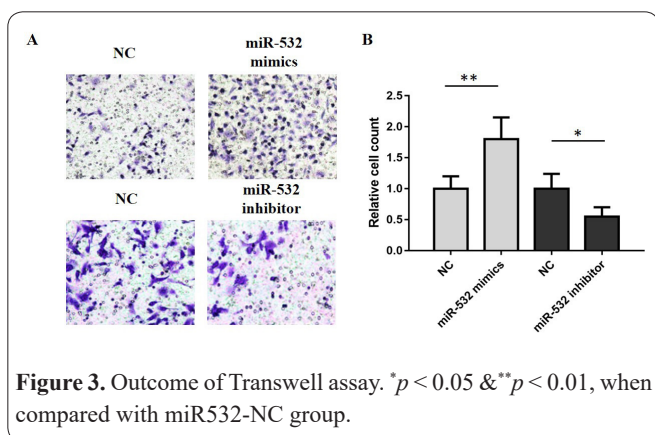


Figure 3. Outcome of Transwell assay. * $p < 0.05$ & ** $p < 0.01$, when compared with miR532-NC group.

of miR532-NC ($p < 0.05$). However, inhibition of miR-532 expression significantly reduced the invasive ability of HCT116 cells ($p < 0.05$). These results are shown in Figure 3.

Outcome of bioinformatics analysis and Western blotting

Results from bioinformatics showed that miR-532 had specific binding sequence with the 3'UTR region of NKD1. After cloning the sequence into the luciferase reporter plasmid, miR-532 significantly inhibited the expression of NKD1 ($p < 0.05$). However, miR-532 had no inhibitory effect on mutated NKD1 3'UTR ($p > 0.05$). The results of Western blotting showed that increased miR-532 expression significantly reduced the expression of NKD1, while decreased miR-532 expression promoted the expression of NKD1 ($p < 0.05$; Figure 4).

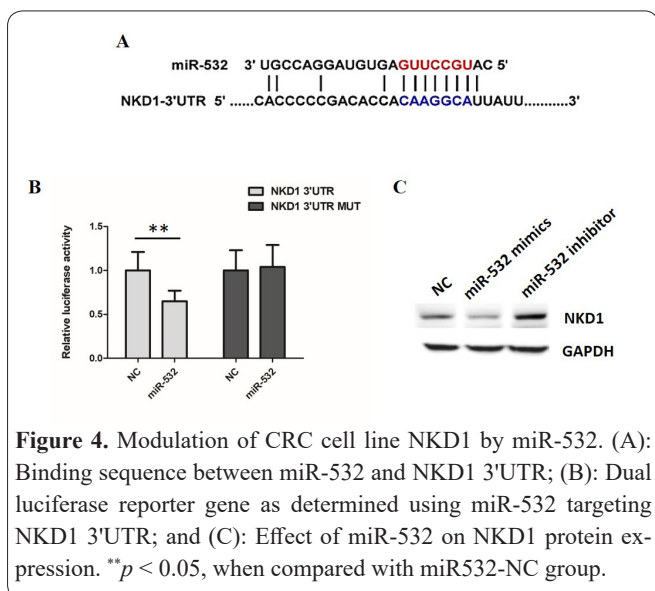


Figure 4. Modulation of CRC cell line NKD1 by miR-532. (A): Binding sequence between miR-532 and NKD1 3'UTR; (B): Dual luciferase reporter gene as determined using miR-532 targeting NKD1 3'UTR; and (C): Effect of miR-532 on NKD1 protein expression. ** $p < 0.05$, when compared with miR532-NC group.

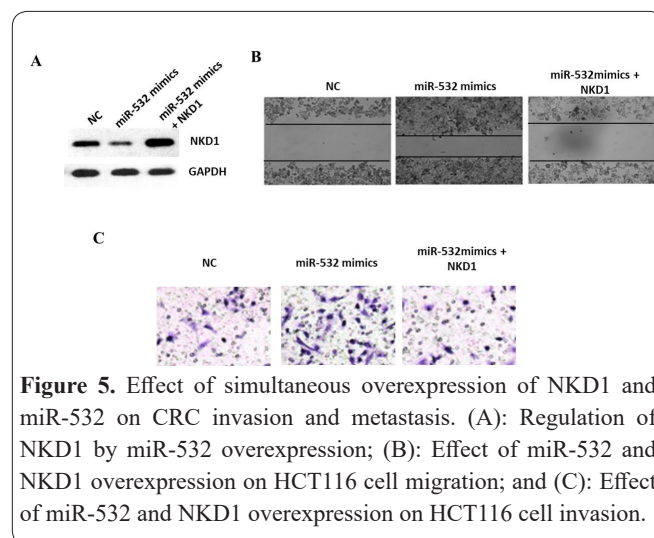


Figure 5. Effect of simultaneous overexpression of NKD1 and miR-532 on CRC invasion and metastasis. (A): Regulation of NKD1 by miR-532 overexpression; (B): Effect of miR-532 and NKD1 overexpression on HCT116 cell migration; and (C): Effect of miR-532 and NKD1 overexpression on HCT116 cell invasion.

Effect of simultaneous overexpression of NKD1 and miR-532 on CRC invasion and metastasis

As shown in Figure 5, overexpression of NKD1 significantly eliminated the inhibitory effect of miR-532 on NKD1 and therefore suppressed cell invasion and metastasis.

Discussion

The last 10 years saw the development of new methods of diagnosis and treatment of CRC due to emergence of molecular markers (9). Studies have shown that specific miRNAs play key roles in tumorigenesis and metastasis. The discovery of tumor-related microRNAs and elucidation of their regulatory mechanisms have helped to unravel their biological roles in the occurrence and metastasis of CRC, and provided new diagnostic and therapeutic targets for CRC treatment.

MicroRNA-532 is abnormally expressed in various cancers such as liver cancer (10), esophageal cancer (11), ovarian cancer (12), and pleural pulmonary blastoma (13). However, the expression and role of miR-532 in CRC have not been reported. The present study investigated the effect of miR-532 on invasion and metastasis of CRC cells, and the underlying mechanism. The results showed that miR-532 acted as a tumor-promoting gene, was highly expressed in CRC cells, and it promoted invasion and metastasis of the cells. The results of bioinformatics revealed a potential binding site for 3'UTR region of NKD1. In this study, NKD1 acted as a tumor suppressor gene, while miR-532 promoted the invasion and metastasis of CRC cells by inhibiting NKD1 expression.

It has been reported that NKD1 inhibits the invasion of non-small cell lung cancer (NSCLC) and that it is a good prognostic factor (14). In addition, NKD1 inhibits the metastasis of liver cancer via inhibition of the expression and activity of Rac1 (15). Naked cuticle homolog 1 (NKD1) is also a prognostic tumor suppressor gene in invasive breast ductal carcinoma (16). In gastric cancer, the Homeobox protein, Hox-A11 (HOXA11) inhibits proliferation, invasion, metastasis, and apoptosis of gastric cancer cells by up-regulating NKD1 (17). In this study, the expression of NKD1 inhibited by miR-532 was significantly up-regulated by NKD1 overexpression, which leads to decreased cell invasion

and metastasis. These results suggest that the level of expression of miR-532 may be positively correlated with tumor metastasis and invasion.

MicroRNA-532 is highly expressed in CRC cells and it is capable of targeting NKD1 expression, thereby promoting invasion and metastasis of the cells. These results suggest that the effect of miR-532 on cell invasion and metastasis may be mediated by NKD1 protein.

MicroRNA-532 is highly expressed in CRC cells. It directly inhibits NKD1 expression and promotes invasion and metastasis of CRC cells. Thus, MicroRNA-532 enhances the development of CRC by inhibiting the expression of NKD1.

Acknowledgement

None.

Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Author's contribution

All work was done by the author s named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhenling Ji; Xujun Song, Rong Wu, Qingsong Tao, Liangtao Zhao, Zhenling Ji collected and analysed the data; Xujun Song wrote the text and all authors have read and approved the text prior to publication.

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