

Original Research Article

MicroRNA-612 regulates the proliferation and epithelial-to-mesenchymal transition of human colon cancer cells via G protein-coupled receptor 132 (GPR132)

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Abstract

Purpose: To investigate the effect of microRNA-612 (miR-612) on human colon cancer cells, and the mechanism involved.

Methods: Expressions of miR-612 and GPR132 were determined by quantitative real-time polymerase chain reaction (qRT-PCR), while cell viability was evaluated using cell counting kit-8 (CCK8) and colony formation assays. Dual luciferase assay was used to determine the interaction between miR-612 and GPR132, while cell migration and invasion were measured by Transwell assay.

Results: The expression levels of miR-612 in colon cancer tissues and cell lines were significantly down-regulated ($p < 0.05$). Overexpression of miR-612 in colon cancer cells led to significant inhibition of their proliferation and colony formation. Transwell assays revealed that miR-612 overexpression markedly stopped the migration, invasion and epithelial-to-mesenchymal transition.

Conclusion: These results indicate that miR-612 exerts anti-cancer effect by suppressing the expression of GPR132 at the translational level. The *in vitro* tumor suppressive activity of miR-612 against colon cancer reveals its potential for the management of colon cancer.

Keywords: Colon cancer, micro-RNA, G-protein coupled receptor, Epithelial-to-mesenchymal transition

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INTRODUCTION

Colon cancer is a malignancy in a part of the large intestine, and it is regarded as the most frequently encountered cancer of the digestive tract [1,2]. Colon cancer is ranked as the third most prevalent cancer in humans, and its mortality makes it the fourth most deadly human cancer [3,4]. Although advanced screening methods for colon cancer have led to

improvements in its diagnosis, the malignancy is still associated with poor survival which largely results from its near-symptomless nature [5]. It has been suggested that colon cancer is triggered by environmental factors and genetic aberrations [6]. Therefore, studies on the pathogenesis of colon cancer at the molecular level might hopefully lead to formulation of better genetic therapeutic approaches against this disastrous neoplasm. In this regard, micro-RNAs (miRs) are promising areas of research. The

miRs are a heterogeneous assemblage of non-coding, single-stranded regulatory RNAs which do not exceed 25 nucleotides in length. They are involved in silencing or repression of specific target genes at post-transcriptional or translational level. They play vital biological roles in various aspects of human body. Thus, impairment of their functions may pave way for a number of diseases, including cancers [7].

It has been reported that dysregulation of miRs may serve as vital cue for intestinal abnormalities, and also play a role in pathogenesis of colon cancer. However, several miRs have been shown to exert tumor suppressive actions against human colon cancer by restricting its progression and metastasis [8]. In the present study, the effect of microRNA-612 (miR-612) on colon cancer was investigated. miR-612 acts as tumor suppressor by controlling the growth and propagation of a number of human cancers via post-transcriptional targeting of different protein-coding genes. For instance, it has been reported that miR-612 inhibited human cervical cancer by targeting NOB1 [9]. Besides, its negative effect on colorectal cancer via miR-612/AKT axis has been demonstrated [10]. However, its effect on colon cancer, and possible link with G-protein coupled receptors (GPRs) have not been studied. In this study, it was found that G-protein coupled receptor 132 (GPR132) was the post-transcriptional target of miR-612. The results also showed that miR-612/GPR132 molecular axis negatively regulated the growth of colon cancer cells, thereby revealing its therapeutic potential against this disease.

EXPERIMENTAL

Clinical tissues, cell lines and cell transfection

A total of 20 colon cancer tissues, together with normal adjacent specimens were taken from colon cancer patients who underwent radical surgical resection at Tongji Medical College, Huazhong University of Science and Technology, Wuhan City, China. The patients ranged in age from 48 to 86 years. All the patients provided written informed consent. This study was approved by the ethics committee of our institution. The excised tissues were preserved at -80°C prior to analysis. Colonic epithelial cell line (CCD841CON) and four colon cancer cell lines (HT29, HCT116, SW620 and T84) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were propagated using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and antibiotic mixture of

penicillin/streptomycin (100 U/mL each) at 37 °C in a humidified 5 % CO₂ incubator. Prior to transfection, the cells were treated with 0.25 % trypsin and plated in 6-well plates at a density of 5×10^4 cells/well. The transfection was carried out using Lipofectamine 2000 (Invitrogen) in line with the manufacturer's protocol. The transfected cells were harvested at 75 - 80 % confluence. The transfection constructs and negative controls were purchased from RiboBio, Gunagzhou, China.

RNA isolation and gene expression analysis

TRIzol reagent (Invitrogen) was used to extract total RNA from tissues and cells. Using RevertAid First Strand cDNA Synthesis kit, the RNA was reverse-transcribed to cDNA. Then real-time PCR was performed using a standard TaqMan based PCR method (Applied Biosystems). The estimation of relative gene expression levels was made using the ΔCT method. The gene expression was normalized to that of β -actin. Three replicates were used per reaction in the qRT-PCRs, and the results were calculated as mean \pm standard deviation.

CCK-8 proliferation assay

The stably transfected cancer cells were retrieved at exponential phase of growth and seeded into 96-well plates at a cellular density of 2.5×10^5 per well. According to the manufacturer's instructions, CCK-8 solution was added (CCK-8 assay kit, Thermo Fisher Scientific) and cell proliferations were determined at different culture durations. The absorbance of each well was measured at 450 nm using a microplate reader (HBA-1096A, DeTie, Shanghai, China).

Clonogenic assay

Exponential growth phase transfected cells were trypsinized, and cell density was adjusted to 300 cells/mL. Cells in 2 mL medium were cultured for 3 weeks using a 6-well plate at 37 °C. The medium was changed twice a week. After 3-weeks, 4 % paraformaldehyde was used for fixing the cells which were then stained with crystal violet. The colonies were visualized under a light microscope and photographed using a camera (Eos RP, Canon, Japan).

Transwell chamber migration and invasion assays

The migration and invasion of stably transfected colon cancer cells were analysed using cell Transwell chamber system (Corning, USA). The

Transwell chambers were fitted with polycarbonate membranes of 8- μ m pore size. For invasion analysis, Matrigel (Corning) precoated membranes were used, but for analysis of cell migration, the membranes were devoid of Matrigel pre-coating. The upper chambers were inoculated with culture medium containing 2.5×10^5 transfected cells, while the lower chambers contained medium supplemented with 10 % FBS only. The cells were incubated at 37 °C for 24 h, after which cells on the upper surface of the membrane were swabbed with cotton, while those that migrated/invaded the lower chambers were fixed in methanol, stained with crystal violet, and examined and counted using an inverted microscope.

MiR target prediction and luciferase reporter assay

Targetscan online tool (http://www.targetscan.org/vert_72/) was used to predict the specific binding target for miR-612, along with retrieval of the actual binding site. The luciferase reporter constructs were constituted using PgL6 plasmid (Beyotime, Shanghai, China). The PgL6GPR132-wt and PgL6-GPR132-mut plasmids carried 3'-UTR stretches of GPR132 with wild type mutated binding site for miR-612. The reporter plasmids were co-transfected into cancer cells with miR-612 mimics or its negative control miR-NC. Then, luciferase activity was assayed using the Dual Luciferase reporter gene assay kit (Beyotime, Shanghai, China) as per the protocol in the user manual.

Statistical analysis

The SPSS 16.0 software (SPSS, Chicago, IL) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA) were used for performing all statistical analyses of the study data. Statistical analysis was done using Student's *t*-test. Values of $p < 0.05$ were taken as indicative of statistically significant differences.

RESULTS

Colon cancer exhibited down-regulated miR-612

The results from qRT-PCR revealed that colon cancer tissues expressed significantly lower miR-612 levels than adjacent non-cancerous colon tissues ($p < 0.05$; Figure 1 A). Moreover, the expression of miR612 was markedly lower in all the colon cancer cell lines (HT29, HCT116, SW620 and T84) than in colon epithelial cells

(Figure 1 B). Among the cancer cell lines, SW620 exhibited the lowest transcript level of miR-612. Therefore, it was used for characterizing the role of miR-612.

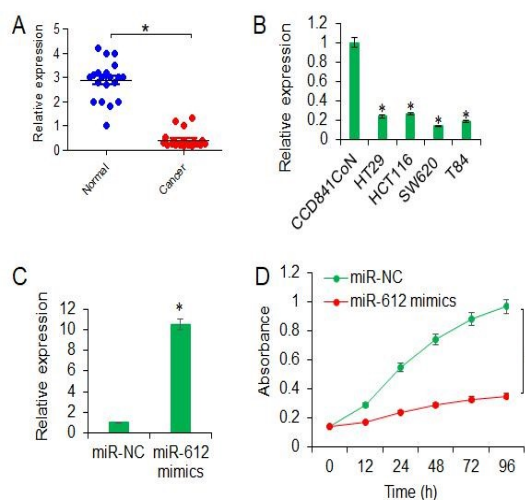


Figure 1: Effect of miR-612 on the proliferation of human colon cancer cells. Expression of miR-612 was determined with qRT-PCR in (A) colon cancer and normal adjacent tissues, and (B) colon cancer and normal cell lines. (C) Expressions of miR-612 in miR-NC and miR-612 transfected SW620 cells. (D) Viability of the miR-NC and miR-612 transfected SW620 cells. The experiments were performed in triplicate, and results are expressed as mean \pm SD. * $p < 0.05$

MiR-612 inhibited proliferation and colony formation of SW620 cells

To find out whether miR-612 regulated proliferation of colon cancer cells, SW620 cells were first transfected with miR-612 mimics (over-expression). The SW620 cancer cells transfected with miR-NC (mimics control) served as negative expression control. Results from RT-PCR showed that miR-612 mimic-transfected cells produced 6-fold miR-612 over-expression, relative to negative control cells (Figure 1 C). The proliferation of miR-612 over-expressing cells was significantly down-regulated when assessed at different culture durations, relative to the negative control cells ($p < 0.05$; Figure 1 D). The effect of miR-612 over-expression on colon cancer cell viability was also determined in terms of colony-forming potential. The relative number of colonies were significantly lower for colon cancer cells over-expressing miR-612 than for respective negative control cells ($p < 0.05$; Figure 2 A). These results are suggestive of anti-proliferative regulatory role of miR-612 in colon cancer.

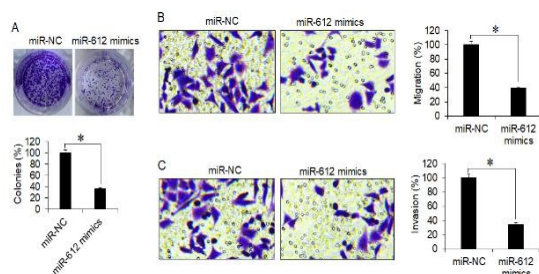


Figure 2: Effect of miR-612 on the migration and invasion of colon cancer cells. (A) Colony formation, (B) migration, and (C) invasion of the miR-NC and miR-612-transfected SW620 cells. ($n=3$; $*p < 0.05$)

MiR-612 suppressed the migration, invasion and epithelial-to-mesenchymal transition of colon cancer cells, and exerted its tumor-suppressive effect by targeting GPR132

Transwell assays were carried out in order to investigate the effect of miR-612 over-expression on the migration and invasion of colon cancer cells *in vitro*. The results revealed that miR-612 over-expression markedly reduced the migration of SW620 cancer cells (Figure 2B). The colon cancer cells migrated at one-third of the migratory rate of negative control cells. Similarly, the invasion of colon cancer cells was significantly reduced under miR-612 up-regulation ($p < 0.05$; Figure 2 C). Cell invasion decreased by about 70 %. Moreover, the epithelial-to-mesenchymal transition of colon cancer cells was significantly decreased ($p < 0.05$), as evidenced by marked increases in the protein expression levels of intracellular epithelial markers (E-cadherin and α -catenin), relative to significant down-regulation in protein expression levels of mesenchymal markers (Snail and vimentin) (Figure 3). These results suggest that miR-612 exhibited negative regulatory effect on the metastatic behaviour of colon cancer cells.

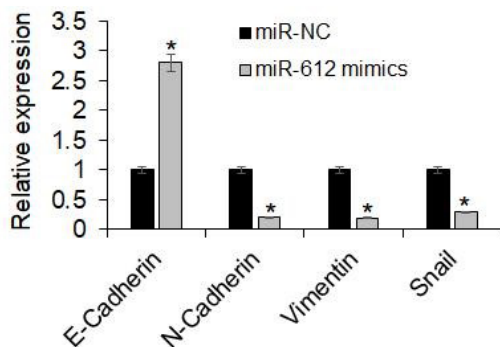


Figure 3: Effect of miR-612 on epithelial-to-mesenchymal transition. Results from qRT-PCR showing the effects of miR-612 on the expressions of E-cadherin, N-Cadherin, Vimentin and Snail. ($n=3$; $*p < 0.05$)

Probable target of miR-612

Online bio-informatics was used for predicting the probable regulatory target of miR-612 in colon cancer cells. It was predicted that miR-612 targeted GPR132 by interacting with a specific nucleotide site in its 3'-UTR in a sequence-specific manner (Figure 4 A). The targeting of GPR132 by miR-612 was confirmed with luciferase reporter assay. The results showed that colon cancer cells co-transfected with 3'-UTR stretch of GPR132 (wt) with miR-612 mimics had significant loss of luciferase activity, when compared to when 3'-UTR of GPR132 was carrying mutated (mut) binding site for miR-612 (Figure 4 B). The qRT-PCR expression analysis of GPR132 also revealed that it was significantly ($p < 0.05$) up-regulated in colon cancer tissues and cancer cell lines (Figure 4 C and D).

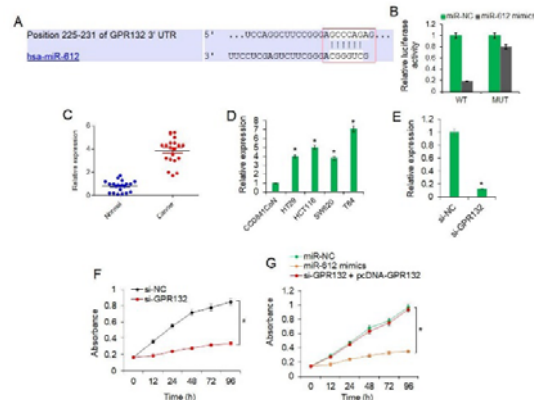


Figure 4: miR-612 exerted its effects by targeting GPR132. (A) TargetScan analysis showing GPR132 as the target of miR-612. (B) Dual luciferase assay showing interaction between miR612 and GPR132. Expression of GPR132 in (C) colon cancer and normal tissues and (D) colon cancer and normal cell lines, and (E) si-NC and si-GPR132 transfected SW620 cells. Cell viability of (F) si-NC and si-GPR132, and (G) miR-NC, miR-612 and si-GPR132 + pcDNA-GPR132 transfected cells. ($n=3$; $*p < 0.05$)

The miR-612 over-expressing SW620 cells expressed significantly lower ($p < 0.05$) GPR132 protein (Figure 4E). In addition, the colon cancer cells subjected to GPR132 silencing via si-GPR132 transfection showed reduced proliferation which mimicked that seen under miR-612 over-expression (Figure 4 F). Conversely, colon cancer cells transfected with over-expression plasmids of GPR132 (pcDNA-GPR132) had good proliferation comparable to that of control colon cancer cells, even under miR-612 over-expression (Figure 4 G). Again, silencing of GPR132 significantly reduced the colony formation of colon cancer cells (Figure 5

A). In addition, colon cancer cell migration, invasion and epithelial-to-mesenchymal transition were inhibited by GPR132 silencing in the same fashion as miR-612 over-expression (Figure 5 B–D). Thus, it is evident that miR-612 exerted its regulatory effect via post-transcriptional silencing of GPR132.

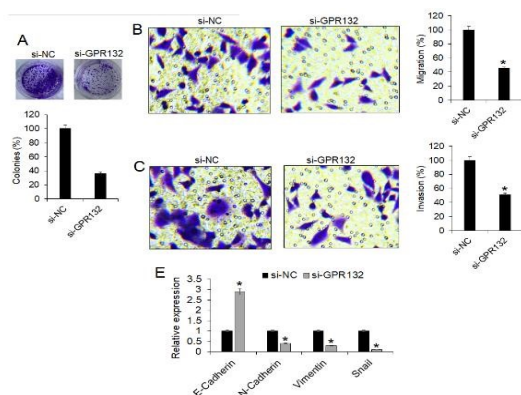


Figure 5: Effect of GPR132 silencing on the migration and invasion of colon cancer cells. (A) Colony formation, (B) migration, and (C) invasion of the si-NC and si-GPR132-transfected SW620 cells. ($n = 3$; $*p < 0.05$)

DISCUSSION

Impairment of the fine regulatory programs in cells has major negative consequences at cellular level and could induce the onset of diseases [11]. Cancer is one of such diseases which may arise as a consequence of cellular disturbance in which the cells evade the programmed molecular course, resulting in unwanted build-up of cellular mass [12]. Aberrant expressions of small regulatory RNAs, i.e., micro-RNAs (miRs) are among molecular changes associated with the pathogenesis and growth of human cancers. These events mark the pathogenesis of almost all human cancers, including colon cancer [13].

Recent studies have demonstrated that dysregulation of miRs is involved in the growth as well as metastasis of human cancers [14]. The present study exemplifies such molecular irregularity by showing that miR-612 expression was significantly reduced in malignant colon tissues and cell lines. Up-regulation of miR-612 has also been reported in other types of human cancers [15].

A number of reports have indicated negative regulatory role of miR-612 in the growth of human cancer cells [16]. Hence, the decline in proliferation of colon cancer cells due to miR-612 over-expression supports the tumor-suppressive

regulatory potential of miR-612 against human colon cancer. Moreover, the results of the present study revealed that colon cancer cell migration and invasion were significantly suppressed by miR-612 over-expression. These results also support the anti-cancer potential of miR-612, thereby reflecting its therapeutic importance.

Migration and invasion are crucial hallmarks of cancer cell metastasis. The latter is favored by the morphological and physiological changes in cancer cells at the molecular level, with epithelial-to-mesenchymal transition as one of these molecular events [17]. During transition from epithelial to mesenchymal state, cancer cells are freed from inter-cellular adhesion, making them attain high motility necessary for cancer invasion [18]. Epithelial-to-mesenchymal transition is regulated by a number of molecular factors, the expression patterns of which are used as indices of this cellular transition [19]. The present study showed that miR-612 exerted negative regulatory effect on epithelial-to-mesenchymal transition of colon cancer cells, which was evident in the down-regulated expression levels of epithelial marker proteins (E-cadherin and α -catenin) and up-regulated expressions of mesenchymal marker proteins (Snail and vimentin) [20]. Multiple regulatory targets which mediate the molecular effect of miR-612 have been identified in human cancers. The G-protein couple receptor 132 (GPR132) was identified as the functional target of miR-612 in colon cancer, as per the results of the present study. The oncogenic role of GPR132 has been established previously [21]. Therefore, the miR-612/GPR132 axis might be utilized as a possible therapeutic target for colon cancer. However, this proposition needs to be supported with *in vivo* studies.

CONCLUSION

The results of the current study indicate that miR-612 is significantly repressed in colon cancer. The tumor-suppressive action of miR-612 against colon cancer cells is evident from its *in vitro* potential to inhibit growth, migration, invasion and epithelial-to-mesenchymal transition of colon cancer cells. Moreover, GPR132 was identified as the specific target and modulator of the regulatory effect of miR-612 on colon cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The experiments were performed by Sha Shen and Qingqing Guo under the supervision of Shuiping Zhan. The funding part was offered by Shuiping Zhan.

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