MicroRNA-214 suppresses the viability, migration and invasion of human colorectal carcinoma cells via targeting transglutaminase 2

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Abstract. Colorectal carcinoma (CRC) is a common malignancy of the digestive tract. MicroRNA (miR)-214 is considered a key hub that controls tumor networks; therefore, the effects of miR-214 on CRC were examined and its target gene was investigated in this study. The expression levels of transglutaminase 2 (TGM2) and miR-214 were detected in CRC and adjacent normal tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting, and luciferase activity was analyzed by dual luciferase reporter analysis. In addition, cell viability, invasion and migration were measured by Cell Counting Kit-8 and Transwell assays, respectively. The expression levels of epithelial-mesenchymal transition-related proteins, and phosphoinositide-3 kinase (PI3K)/protein kinase B (Akt) signaling-associated factors were detected using RT-qPCR and western blotting. The results demonstrated that miR-214 expression was downregulated in CRC tissue, whereas TGM2 expression was upregulated. According to TargetScan prediction, miR-214 possesses a binding site to TGM2. In addition, transfection with miR-214 mimics markedly suppressed the viability of Lovo cells. miR-214 overexpression also inhibited cell invasion and migration by decreasing E-cadherin and tissue inhibitor of metalloproteinase (MMP)-2 and MMP-9 expression. Furthermore, miR-214 downregulated phosphorylation of PI3K and Akt; however, the expression levels of total PI3K and Akt were not affected by miR-214. In conclusion, this study indicated that TGM2 was a target gene of miR-214, and a negative correlation between miR-214 and TGM2 expression was determined in CRC. Notably, miR-214 markedly suppressed the viability, invasion and migration of CRC cells, which may be associated with a downregulation in PI3K/Akt signaling. These findings suggested that miR-214 may be considered a novel target for the treatment of CRC.

Introduction

Colorectal carcinoma (CRC) is a common malignancy of the digestive tract (1), the incidence of which is increasing annually in China (2). If patients with CRC metastasis in advanced stages are not promptly treated, their average survival is only 5-6 months (3,4), and the main causes of CRC-associated mortality are invasion and metastasis (5,6). Therefore, it is particularly important to identify novel tumor markers to inhibit tumor metastasis.

In the occurrence and development of invasive carcinoma from atypical hyperplasia and carcinoma in situ, the destruction of epithelial integrity is an important event (7). The first stage of tumor invasion and metastasis includes destruction of the intact epithelial structure and acquisition of stromal cell characteristics; this process is known as epithelial-mesenchymal transition (EMT). EMT is a phenomenon during which epithelial cells convert to interstitial cells under specific physiological and pathological conditions (8). It has been reported that EMT is closely associated with tumor invasion and metastasis, and that it serves a key role in in situ infiltration and distant metastasis of various types of cancer (9,10). Various cancer cells can undergo partial or complete EMT (11-13).

MicroRNAs (miRNAs/miRs) are a series of endogenous non-coding small RNA molecules, usually 18-25 nt in length (14). miRNAs suppress protein translation through binding to the 3' untranslated region (3'UTR) of target gene mRNA (15,16), miRNAs serve a key role in translation inhibition following gene transcription. In recent years, increasing experimental evidence has demonstrated that abnormal miRNA expression in tumor cells is closely associated with the occurrence of tumors. Different degrees of abnormal miRNA expression (17-20) can be detected in all types of human cancer, including CRC. In addition, miRNAs have been reported to exert a strong regulatory effect on EMT (21-23). Notably, miR-214 is considered a key hub that controls tumor networks (24); however, the role and mechanism of miR-214 in the development of CRC are currently unclear.

In the present study, the expression of miR-214 was detected in CRC tissues, and its target gene was identified. Furthermore, the effects of miR-214 on viability and motility...
of CRC cells were determined, and the underlying molecular mechanism was analyzed.

**Materials and methods**

**Tissue source.** Between November 2016 and December 2017, 36 CRC and adjacent normal tissues were collected from patients with CRC that were admitted to The Affiliated Dongtai Hospital of Nantong University (Dongtai, China). Written informed consent was obtained from patients permitting their tissues to be used. The present study was approved by the Ethics Committee of The Affiliated Dongtai Hospital of Nantong University (approval no. 201501218).

**Cell line.** The LoVo human colon adenocarcinoma cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in RPMI-1640 medium (M&C Gene Technology Co., Ltd., Beijing, China) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an incubator containing 5% CO₂.

**Cell transfection.** miR-214 mimics and miRNA mimics control were purchased from Genomeditech (Shanghai, China). miR-214 mimics sense, 5’-ACGACGGACGACAGGCGAGU-3’, and antisense, 5’-UGCCUGUCUGCCCUAGCGUUU-3’; mimics control sense, 5’-UUCUCCGAACGUGACACGU-3’, and antisense, 5’-ACGUGACACGUUCCAGAATT-3’. Using Lipofectamine® 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), miR-214 mimics (50 nM; mimic group) or miRNA mimics control (50 nM; mock group) were transfected into LoVo cells (60-80% confluence) at 37°C for 24 h, and the control group were treated with PBS. In addition, cells were treated with 50 ng/ml insulin-like growth factor-1 (IGF-1; AmyJet Scientific, Wuhan, China) for 24 h at 37°C, in order to activate phosphoinositide 3-kinase (PI3K) (25).

**Dual luciferase reporter assay.** TargetScan version 7.2 (http://www.targetscan.org/vert_72/) was used to predict the binding site between the 3’-UTR of transglutaminase 2 (TGM2) and miR-214. For the dual luciferase reporter experiments, the 3’-UTR of TGM2 gene and a mutant (mut) 3’-UTR of TGM2, and mir-214. For the dual luciferase reporter experiments, the binding site between the 3’-UTR of transglutaminase 2 (TGM2) (http://www.targetscan.org/vert_72/) was used to predict the Dual luciferase reporter assay. TargetScan version 7.2

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was extracted from cells and tissues using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT was conducted to synthesize cDNA from 2 µg RNA using H BeyoRT II First Strand cDNA Synthesis kit (Beyotime Institute of Biotechnology, Haimen, China), according to manufacturer's protocol. The primer sequences are listed in Table I. cDNA was amplified using SYBR Green qPCR Master Mix (MedchemExpress, Monmouth Junction, NJ, USA). The conditions of amplification were as follows: Pre-denaturation at 95°C for 10 sec, followed by 30 cycles of denaturation at 95°C for 5 sec and 62°C for 25 sec, and a final elongation step at 70°C for 30 min. The internal controls were U6 and GAPDH. Gene expression was calculated and quantified using the 2⁻ΔΔCT method (26).

**Western blot analysis.** Total proteins were extracted from cells and tissues using radioimmunoprecipitation assay buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Protein concentration was analyzed using Pierce Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). The protein lysate (25 µg) was then separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequently, 5% non-fat milk was applied to block the membrane at 37°C for 60 min, and the membrane was incubated with anti-TGM2 (cat. no. ab216018, 1:800; Abcam, Cambridge, MA, USA), anti-tissue inhibitor of metalloproteinasases-2 (TIMP-2; cat. no. ab180630, 1:1,000; Abcam), anti-matrix metalloproteinasse (MMP)-2 (cat. no. ab37150, 1:200; Abcam), anti-MMP-9 (cat. no. ab73734, 1:600; Abcam), anti-E-cadherin (cat. no. ab15148, 1:800; Abcam), anti-phosphorylated (p)-PI3K (cat. no. ab38364, 1:600; Abcam), anti-PI3K (cat. no. MAB2686, 1:600; R&D Systems, Inc.), anti-p-protein kinase B (Akt; cat. no. MAB887, 1:800; R&D Systems, Inc.), anti-Akt (cat. no. MAB2055, 1:800; R&D Systems, Inc.) and anti-GAPDH (cat. no. ab181602, 1:600; Abcam) at 4°C overnight. The membrane was then incubated with corresponding secondary antibodies [goat anti-mouse immunoglobulin (Ig) G H&L, cat. no. ab6708, 1:6,000; goat anti-rabbit IgG H&L (horseradish peroxidase), cat. no. ab6721, 1:7,000; Abcam] at 37°C for 60 min. The proteins were visualized using an enhanced chemiluminescence system (GE Healthcare, Chicago, IL, USA). The protein levels were quantified using Bio-Rad ChemiDoc system with Image Lab software (version 6.0 Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell Counting kit-8 (CCK-8) analysis.** LoVo cells were inoculated in a 96-well plate (3x10⁴ cell/well) in an incubator at 37°C for 24 h. After culturing, cells were treated with PBS (control group), or were transfected with miRNA mimics (mock group) or miR-214 mimics (mimics group) for 48 h. Subsequently, 10 µl CCK-8 reagent (Beyotime Institute of Biotechnology) was then transferred into a black enzyme plate. Then, the Luciferase assay reagent II (100 µl) and 1X Stop&Glo® reagent (100 µl) (Promega Corporation) were added to the cells, and luciferase activity was detected using the GloMax® Discover Multimode Microplate Reader (cat. no. GM3000; Promega Corporation) according to the manufacturer's instructions. Luciferase activity was normalized to Renilla luciferase.
was added into each well, and cells were incubated with CCK-8 reagent for 4 h at 37°C. Cell viability was determined at 450 nm using a microplate absorbance spectrophotometer (Bio-Rad Laboratories, Inc.).

Transwell analysis. Migration and invasion of cells were determined using Transwell analysis. Cell invasion was measured using Matrigel-coated Transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA), and cell migration was measured using uncoated Transwell chambers (BD Biosciences). Invasion assay was performed using Transwell inserts pre-coated with Matrigel (BD Biosciences). RPMI-1640 medium supplemented with 12% FBS was added to the lower chamber. The treated cells were digested with trypsin and a cell (2x10^5 cells/ml) suspension was incubated in the upper chamber at 37°C for 24 h. Subsequently, the cells were fixed with 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet for 20 min at room temperature. The stained cells were observed under a light microscope (magnification, x200).

Statistical analysis. SPSS 20.0 software was used to conduct statistical analysis. Data are presented as the means ± standard deviation. Differences among the groups were analyzed by Tukey test. Differences between two groups were analyzed by Student’s t-test. The association between TGM2 expression and the clinicopathological features of patients with CRC was investigated using χ² test. The correlation between miR-214 and TGM2 expression in CRC was determined using the two-tailed Spearman nonparametric correlation test. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated independently at least three times.

### Results

TGM2 was the target gene for miR-214 in CRC. The expression levels of miR-214 and TGM2 were detected in CRC and adjacent normal tissues using RT-qPCR and western blotting. Compared with adjacent normal tissues, the expression levels of miR-214 were decreased in CRC tissues; however, TGM2 expression was increased (Fig. 1a-c). Two-tailed Spearman nonparametric correlation test identified a negative correlation between miR-214 and TGM2 expression in CRC tissues (Fig. 1d). When LoVo cells were transfected with miR-214 mimics, miR-214 expression was enhanced, whereas the mRNA and protein expression levels of TGM2 were suppressed (Fig. 1e-G). According to TargetScan (http://www.targetscan.org/vert_72/), miR-214 possessed TGM2 binding sites (Fig. 1H); therefore, the binding abilities of miR-214 and TGM2 were tested using a dual luciferase reporter assay. Although relative luciferase activity was reduced in cells exposed to miR-214 mimics and TGM2-3’UTR, it remained relatively stable in cells co-transfected with miR-214 + TGM2-3’UTR mut (Fig. 1I). Furthermore, TGM2 expression was divided into a low expression group and a high expression group according to the median value of relative expression, and the results of a χ² test revealed that TGM2 expression was associated with lymph node metastasis; however, it was not associated with age, sex, tumor location or tumor type (Table II).

### miR-214 suppresses the viability, migration and invasiveness of LoVo cells. CCK-8 and Transwell assays were performed to explore the effect of miR-214 on the viability, migration and invasion of LoVo cells. CCK-8 results revealed that miR-214 overexpression inhibited cell viability compared with in the

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| Primer name          | Sequence (5'-3')                  | Product size (bp) |
|----------------------|-----------------------------------|-------------------|
| miR-214-Forward      | ATAGAATTCTTCTTCTCCCTTTCCCTTACTTCC |                  |
| miR-214-Reverse      | CCAGGATCTTCTTCTAGCCACCTCACCTTAA   | 235               |
| TGM2-Forward         | CCGAGAGGCTTGGTCTTAAAG            | 236               |
| TGM2-Reverse         | TCTTAGTGAAAAACGGGCCT              |                  |
| E-cadherin-Forward   | TTTGAAGATGCAACCAGGTCTG           |                  |
| E-cadherin-Reverse   | CAGCGGTACCTTTGGTGAAA             | 180               |
| TIMP-2-Forward       | AGCACACCAGCAAGAAAGAG             |                  |
| TIMP-2-Reverse        | TGAAGAGGACCAAGAAACTTGG           | 175               |
| MMP-2-Forward        | TGCTACACACCTGATCTGG              |                  |
| MMP-2-Reverse        | GATGACGTCCCACCACGTCACCA          | 184               |
| MMP-9-Forward        | GAGACTCTACACCCAGAGAGC            |                  |
| MMP-9-Reverse        | GAAAGTGAAAAGGAAAGACGC            | 238               |
| U6-Forward           | CTCGCTTCGGCACAGCA                |                  |
| U6-Reverse           | AACGCTTCACAGAATTGGGT             | 215               |
| GAPDH-Forward        | CCATCTTCCAGAGCGAGAT              |                  |
| GAPDH-Reverse        | TGCTGATGATCTTTGAGGCTG            | 222               |

miR-214, microRNA-214; MMP, matrix metalloproteinase; TGM2, transglutaminase 2; TIMP-2, tissue inhibitor of metalloproteinases-2.
mock group (Fig. 2A). Transwell results demonstrated that when cells were exposed to miR-214 mimics, the relative rates of migration (Fig. 2B) and invasion (Fig. 2C) were markedly reduced compared with in the mock group.

miR-214 regulates EMT-associated factors in LoVo cells. To examine the molecular mechanism underlying the effects of miR-214 on cell migration and invasion, the expression levels of EMT-associated factors (TIMP-2, MMP-2, MMP-9 and E-cadherin) were detected by RT-qPCR and western blotting. As determined by RT-qPCR, the expression levels of TIMP-2 and E-cadherin were enhanced in the mimics group; however, MMP-2 and MMP-9 expression was inhibited compared with in the mock group (Fig. 3 a-d). In addition, as determined by western blotting, miR-214 overexpression significantly suppressed the protein expression levels of MMP-2 and MMP-9, but promoted TIMP-2 and E-cadherin expression (Fig. 3e).

miR-214 blocks the PI3K/Akt signaling pathway in LoVo cells. In order to assess the effects of miR-214 on signaling in LoVo cells, the PI3K/Akt signaling pathway was analyzed by western blotting. The results revealed that phosphorylation of PI3K and Akt were significantly reduced when cells were exposed to miR-214 mimics, compared with in the control and mock groups (P<0.01). Nevertheless, the protein expression levels of total PI3K and Akt remained stable in all groups (Fig. 4A and B). In addition, the PI3K activator IGF-1 was used to further detect the role of PI3K/Akt signaling; the results revealed that MMP-9 expression was upregulated, whereas TIMP-2 expression was decreased in the IGF-1 + mimic group compared with in the mimic group. These findings indicated that the effects of miR-214 on cell invasion and migration may be partly associated with inhibition of PI3K/Akt signaling (Fig. 5).

Discussion

miR-214 has been reported to be particularly active in cancer, being abnormally expressed in several types of malignant tumors (27-29). However, the expression of miR-214 varies in different tumors, and has certain tumor specificity. Specifically, miR-214 has been demonstrated to have a high expression in pancreatic cancer, oophoroma and melanoma (30-32), and a low expression in esophageal squamous cell carcinoma, liver cancer, breast cancer, cervical carcinoma and CRC (33-37). In the present study, low miR-214 expression was detected in CRC tissues, which was in accordance with the results of previous studies (33,38).

As a member of the transglutaminase family, TGM2 is composed of 687 amino acid residues, and is a calcium-dependent multifunctional protein with a molecular weight of 78 kD (39). Numerous studies have demonstrated that TGM2 expression is closely associated with the proliferation and metastasis of various malignant tumor cells (40-43). TGM2 expression is increased in breast cancer, ovarian cancer, lung cancer and CRC (44-47). Similar to previous findings (45), the present data also demonstrated that TGM2 expression was increased in CRC tissues. It is well known that different miRNAs can regulate the same mRNA molecule, and that the same miRNA can regulate numerous mRNA molecules. In this study, the results of a two-tailed Spearman nonparametric correlation test identified a negative correlation between miR-214 and TGM2 expression in CRC. Furthermore, overexpression of miR-214 significantly inhibited the expression levels of TGM2. Therefore, it may be speculated that TGM2 acts as

### Table II. Association between TGM2 expression and the clinicopathological features of patients with colorectal cancer.

| Variables               | Low TGM2 expression (n=17) | High TGM2 expression (n=19) | P-value |
|-------------------------|----------------------------|-----------------------------|---------|
| Age (years)             |                            |                             | 0.738   |
| <60                     | 8                          | 10                          |         |
| ≥60                     | 9                          | 9                           |         |
| Sex                     |                            |                             | 0.549   |
| Male                    | 10                         | 13                          |         |
| Female                  | 7                          | 6                           |         |
| Tumor location          |                            |                             | 0.955   |
| Colon                   | 10                         | 11                          |         |
| Rectum                  | 7                          | 8                           |         |
| Tumor size              |                            |                             | 0.271   |
| <30 mm                  | 15                         | 14                          |         |
| ≥30 mm                  | 2                          | 5                           |         |
| Lymph node metastasis   |                            |                             | 0.019*  |
| No                      | 12                         | 6                           |         |
| Yes                     | 5                          | 13                          |         |

*P<0.05, χ² test.
a target gene for miR-214 in CRC. According to TargetScan, miR-214 possesses a binding site to TGM2. Additionally, luciferase activity was reduced in cells co-transfected with miR-214 + TGM2-3'UTR; however, it remained stable in cells co-transfected with miR-214 + TGM2-3'UTR mut compared with in the control + TGM2-3'UTR group. These results confirmed that TGM2 was a target of miR-214.

Previous studies have reported that abnormal miRNA expression exists in several human diseases, and that it serves a pivotal role in the occurrence, development, invasion, metastasis and angiogenesis of cancer (15,18-20). Furthermore, miR-214 participates in the growth, invasion and metastasis of cancer. Zhao et al (49) also revealed that miR-214 inhibits the growth and metastasis of lung cancer cells, and Schwarzenbach et al (35) demonstrated that miR-214 inhibits the proliferation and metastasis of cervical cancer and CRC cells. Similarly, the present data demonstrated that miR-214 markedly suppressed the viability, invasiveness and migration of LoVo cells. Notably, decreased cell invasion and migration may be partially caused by inhibited cell viability. 

Li et al (50) reported that decreased expression of miR-214 promotes intrahepatic cholangiocarcinoma metastasis via regulating EMT-related factors (Twist and E-cadherin). Cai et al (51) also demonstrated that miR-194 increases cell metastasis and modulates the EMT process by reducing E-cadherin levels and increasing MMP-2 levels in cancer.
Another study observed that miR-29b inhibits EMT via affecting the expression of E-cadherin, MMPs and TIMPs (52). Therefore, it was hypothesized that miR-214 may suppress the invasion and migration of LoVo cells via regulating EMT-associated factors (E-cadherin, MMPs and TIMPs). As expected, this study revealed that elevated expression of miR-214 markedly increased the expression levels of TIMP-2 and E-cadherin, and reduced MMP-2 and MMP-9 expression. The results suggested that miR-214 inhibited cell invasion and migration through downregulating...
MMP-2 and MMP-9 expression and by upregulating TIMP-2 and E-cadherin expression.

The PI3K/Akt signaling pathway has been increasingly studied in CRC. The pathway serves a critical role in the development of CRC and may be used for the development of novel drugs. PI3K/Akt pathway may potentially be used as a drug target for CRC, as pathway inhibitors may promote apoptosis and inhibit proliferation of CRC cells. Activation of this pathway can inhibit the apoptosis of CRC cells, and promote cell proliferation, invasion and metastasis (53-55). Previous have reported that Gdc-0941, a novel class I PI3K inhibitor, can enhance the efficacy of docetaxel by increasing drug-induced apoptosis in breast cancer models (56). Abubaker et al (53) reported that activation of the PI3K/Akt pathway stimulates cell growth in CRC. Song et al (55) demonstrated that miR-532 attenuates PI3K/Akt signaling to suppress the progression of CRC (55). Jia et al (57) confirmed that miR-182 and miR-135b suppress cell proliferation and motility through inhibiting the PI3K/Akt pathway in CRC. Therefore, in this study, the effects of miR-214 on the PI3K/Akt pathway were examined in LoVo cells via western blotting. The results confirmed that miR-214 suppressed activation of PI3K/Akt signaling. Furthermore, activation of PI3K/Akt reversed the effects of mir-214 on the expression levels of MMP-9 and TIMP-2. Therefore, these findings suggested that inhibition of the PI3K/Akt pathway may be associated with the antitumor effect of miR-214.

In conclusion, the present results demonstrated that TGM2 was a target gene for miR-214, and that a negative correlation existed between miR-214 and TGM2 expression in CRC. miR-214 markedly suppressed the viability, migration and invasion of CRC cells, which was associated with downregulation of the PI3K/Akt signaling pathway. These findings indicated that miR-214 may be considered a novel target for CRC therapy.
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Availability of data and materials

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

Authors' contributions

HS wrote the manuscript. HS, XFZ and CJC performed the experiments and data analysis. HS and CJC designed the study and revised the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of The Affiliated Dongtai Hospital of Nantong University. Patients provided written informed consent permitting their tissues to be used.

Patient consent for publication

Informed consent was obtained from all participants for the publication of their data.

Competing interests

The authors declare that they have no competing interests.

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