MicroRNA-214 promotes the proliferation, migration and invasion of gastric cancer MKN28 cells by suppressing the expression of Dact2

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Abstract. The present study examined the expression of Dapper, antagonist of β-catenin 2 (Dact2) and microRNA (miR)-214 in gastric cancer at tissue and cellular levels, and to understand their biological roles. A total of 42 gastric cancer patients were enrolled in the present study. Bioinformatics tool was used to predict the miR molecule that potentially regulates Dact2 expression. To measure the expression of miR-214 and Dact2, reverse transcription-quantitative polymerase chain reaction was employed. Mixed gastric adenocarcinoma type MKN28 cells were transfected with negative control (NC), miR-214 mimics or inhibitor. The CCK-8 assay was used to investigate the proliferation of mixed gastric adenocarcinoma type MKN28 cells. To study migration and invasion abilities of mixed gastric adenocarcinoma type MKN28 cells, the Transwell assay was performed. To determine the expression of Dact2 protein, western blotting was conducted and the rescue assay was utilized to study the biological roles of miR-214 and Dact2 in mixed gastric adenocarcinoma type MKN28 cells. To test whether Dact2 is a direct target of miR-214, the dual luciferase reporter assay was performed. Results indicated that the expression of miR-214 was elevated, but expression of Dact2 mRNA was decreased in gastric cancer tissues, which was closely correlated with the invasion, metastasis, occurrence and development of gastric cancer. Notably, miR-214 promoted the proliferation of mixed gastric adenocarcinoma type MKN28 cells *in vitro*, whereas but Dact2 inhibited the proliferation of these cells. Downregulation of miR-214 expression or upregulation of Dact2 expression inhibited the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells. Furthermore, miR-214 regulated the expression of Dact2 protein and its downstream β-catenin protein in mixed gastric adenocarcinoma type MKN28 cells. Dact2 reversed the effect of miR-214 on the proliferation, migration and invasion of mixed gastric adenocarcinoma type MKN28 cells. In addition, miR-214 directly targeted the 3'-UTR seeding region of Dact2 mRNA to regulate its expression. The present study demonstrated that expression of miR-214 was upregulated in gastric cancer tissues, and positively correlated with lymphatic metastasis and clinical staging. In addition, expression of Dact2 was downregulated in gastric cancer tissues and negatively correlated with lymphatic metastasis and clinical staging. Notably, the present findings suggest that miR-214 promoted the proliferation, migration and invasion of mixed gastric adenocarcinoma type MKN28 cells by suppressing the expression of Dact2.

Introduction

Gastric cancer is the most common malignant tumor of digestive tract, and its morbidity and mortality are the second highest in all malignant tumors in the world (1-3). After clinical treatments, the five-year survival rate of patients with early gastric cancer can reach 98%, but patients with advanced gastric cancer have poor prognosis due to distant metastasis (4). Tumor recurrence and metastasis are the major causes of death in patients with gastric cancer (5). Because the early symptoms of gastric cancer are not obvious and the disease lack of specific diagnostic markers, most patients are in the middle and late stage of gastric cancer at diagnosis, and postoperative recurrence and metastasis are easy to occur (6). Therefore, researches on the molecular mechanism of recurrence and metastasis of gastric cancer is of great significance for its clinical diagnosis and treatment.

Dapper, antagonist of β-catenin (Dact) gene is a signal pathway regulating molecule that exerts its function by negatively regulating Wnt/β-catenin and Nodal/TGFβ signaling pathways (7,8). Wnt/β-catenin and Nodal/TGFβ signaling pathways participate in the regulation of the occurrence and development of tumors, and Dact gene can therefore play a role in the occurrence and development of tumors by affecting these signaling pathways (9). Dact2 gene is a member of Dact family that plays a role in tumors by participating in the negative regulation of both Wnt/β-catenin and Nodal/TGFβ

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signaling pathways (10). As a tumor-suppressor gene in esophageal cancer, liver cancer, and lymphoma, Dact2 inhibits the occurrence and development of tumors by regulating the methylation of gene promoters (11-13). However, it is still unknown whether Dact2 is regulated by other factors. A study on the methylation in hepatocellular carcinoma and gastric cancer tissues shows that promoter methylation is not found in some patients with low expression of Dact2 (14), suggesting that Dact2 may be regulated by other factors.

MicroRNA (miRNA or miR) is a class of small non-encoding RNA molecules (18-22 nucleotides) that bind with the 3'-untranslated region (UTR) of mRNA to inhibit its translation (15). miRNA plays important regulatory roles in post-transcriptional levels of genes, and widely participates in the proliferation, aging, apoptosis, migration, differentiation, and drug resistance of cells (16). Studies show that the expression of a variety of miRNA molecules is abnormal in gastric cancer, and closely related with the occurrence and development of the disease. For example, miR-1 and miR-200c act as tumor-suppressor genes in gastric cancer, and the downregulation of their expression promotes the migration of gastric cancer cells (17,18). In addition, miR-33b-5p enhances the sensitivity of gastric cancer cells to chemotherapeutic drugs and improves their clinical efficacy (19). Of note, Wnt/β-catenin signaling pathway that is regulated by Dact2, is also regulated by various miRNA molecules (20). Therefore, miRNA is likely to be involved in the regulation of Dact2 gene. In the present study, we investigate the miRNA molecule that regulates Dact2 gene at tissue and cellular levels, and try to elucidate the mechanism of action of the miRNA molecule.

Patients and methods

Bioinformatics. Using miRNA molecule online prediction software Targetscan 7.1 (www.targetscan.org/vert_71/), we predicted the miRNA molecules that might regulate the expression of Dact2 gene following the instructions on the website.

Patients. A total of 42 patients who received surgical resection of gastric cancer tissues at our hospital between December 2014 and February 2016 were included in the present study (Table I). The 42 cases of resected gastric cancer tissues were sliced and stained with hematoxylin and eosin, and examined by two pathologists independently. Patients with lymphatic metastasis were included into N1 group, while those without lymphatic metastasis were included into N0 group. According to 2003 WHO cancer classification criteria, 17 cases were included into stage I, 12 cases were included into stage II, 10 cases were included into stage III, and 3 cases were included into stage IV. Among all 42 patients, 31 patients had moderate or high differentiation, and 11 patients had low differentiation. Tumor-adjacent tissues 5 cm away from tumor tissues were also collected as controls. The tissue samples were frozen in liquid nitrogen and stored at -80˚C. Clinical information and pathological data of the patients were collected. All procedures were approved by the Ethics Committee of Harbin Medical University. Written informed consents were obtained from all patients or their families.

Table I. The clinic characteristics of 42 gastric cancer patients.

Indexes	Number of patients
Sex	
Male	27
Female	15
Age (year)	
≤60	26
≥60	16
Tumor size (cm)	
≤5	30
≥5	12
TNM stages	
I	17
II	12
III	10
IV	3
Differentiation	
Well	16
Moderate	15
Poor	11
Lymphatic metastasis	
Yes	23
No	19

Cells. Mixed gastric adenocarcinoma type Gastric cancer MKN28 line was a derivative of MKN74 cells (which are also a gastric adenocarcinoma cell line) (21). was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2. When reaching 80-90% confluency, the cells were passaged. The medium was replaced every two days. Cells with passage numbers 3-6 were used for experiments.

Mixed gastric adenocarcinoma type MKN28 cells were divided into negative control (NC) group, miR-214 mimics group and miR-214 inhibitor group. On the day before transfection, mixed gastric adenocarcinoma type MKN28 cells ($2x10^5$) in log-phase growth were seeded onto 24-well plates containing antibiotics-free RPMI-1640 medium supplemented with 10% FBS. When reaching 70% confluency, 1.5 μl miR-214 mimics/inhibitor (20 pmol/μl; RiboBio, Guangzhou, China) and 1 μl Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added into two individual vials containing 50 μl Opti Memi medium, respectively. Five min later, the liquids in the two vials were mixed together before standing still for another 20 min. Then, the mixture was added onto the cells for an incubation of 6 h before changing to RPMI-1640 medium supplemented with 10% FBS. The cells were cultured at 37˚C and 5% CO_2 for 48 h before use.

For rescue experiments, mixed gastric adenocarcinoma type MKN28 cells ($2x10^5$) in miR-NC and miR-214 inhibitor groups were seeded into 24-well plates containing antibiotics-free RPMI-1640 medium supplemented with 10% FBS. When reaching 60% confluency, mixed gastric adenocarcinoma type MKN28 cells in miR-214 inhibitor group were

infected by sh-DACT2 plasmid (Hanbio Biotechnology Co., Ltd., Shanghai, China), while cells in miR-NC group were infected by 0.5 μg NC plasmid. After being cultured at 37˚C and under 5% CO_2 for 6 h, the medium was refreshed to newly made RPMI-1640 medium containing 10% FBS before cultivation for 72 h. Then, RPMI-1640 medium containing 1 μg/ml puro was added before incubation for 72 h.

Quantitative real-time polymerase chain reaction (qRT-PCR). Gastric cancer and tumor-adjacent tissues (100 mg) were ground into powder using liquid nitrogen before addition of 1 ml TRIzol isolation reagent (Thermo Fisher Scientific, Inc.) for lysis. After lysis, total RNA was extracted using phenol chloroform method. The purity of RNA was determined by A260/A280 using ultraviolet spectrophotometry (Nanodrop ND2000; Thermo Scientific, Inc.). Then, cDNA was obtained by reverse transcription using miScript II RT kit (Qiagen, Hilden, Germany) from 1 μg RNA and stored at -20˚C.

qRT-PCR was performed using miScript SYBR® Green PCR kit (Qiagen) and the reaction system was composed of 10 μl qRT-PCR-Mix, 0.5 μl upstream primer (5'-ACAGCA GGCACAGACAGGCAGT-3'), 0.5 μl downstream primer (universal primer provided by the kit), 2 μl cDNA and 7 μl ddH_2O. Reaction protocol was initial denaturation at 95˚C for 10 min, and 40 cycles of 95˚C for 1 min and 60˚C for 30 sec.

Cell-Counting kit (CCK)-8 assay. The sample cells were inoculated in 96-well plates at a density of 2,000/well. At 0, 24, 48 and 72 h, 20 μl CCK-8 (5 g/l; Beyotime Institute of Biotechnology, Beijing, China) was added onto the cells. After being incubated at 37˚C for 2 h, absorbance (490 nm) of each well was determined, and cell proliferation curves were plotted. Each group was tested in 3 replicate wells and the values were averaged.

Transwell assay. Matrigel chambers (Corning Inc., Corning, NY, USA) were used to determine the migration and invasion abilities of cells. Matrigel was first diluted with serum-free RPMI-1640 medium at a ratio of 1:2. In upper chamber, 50 μl diluted Matrigel was added and kept at 37˚C for 1 h. Then, $1x10^5$ cells and 200 μl serum-free RPMI-1640 medium were added into the upper chamber. In the lower chamber, 500 μl RPMI-1640 medium supplemented with 10% FBS was added. After incubation for 24 h, the cells in upper chamber were wiped by cotton swab. Then, the chamber was fixed using 4% formaldehyde for 10 min at room temperature, and then subjected to Giemsa's staining for 1 min. After washing for 3 times, cells that moved to the other side of the chamber were counted under a microscope (5 fields; magnification, x200) to evaluate migration and invasion abilities.

Western blot analysis. Cells in each group were trypsinized and collected. Then, cold Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; Beyotime Institute of Biotechnology) was mixed with the samples. Then, the mixture was lysed for 30 min on ice, and then centrifuged at 12,000 rpm and 4˚C for 10 min. Bicinchoninic acid (BCA) protein concentration determination kit (RTP7102; Real-Times Biotechnology Co., Ltd., Beijing, China) was used to determine protein concentration in the supernatant. After mixing protein samples (6 μl) with 5X sodium dodecyl sulfate loading buffer, the mixture was denatured by boiling in water bath for 10 min. Afterwards, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (100 V) was performed using the samples. Then, the proteins were electro-transferred to polyvinylidene difluoride (PVDF) membranes on ice (250 mA, 1 h) before being blocked with 50 g/l skimmed milk at room temperature for 1 h. Afterwards, rabbit anti-human β-catenin and DACT2 polyclonal primary antibodies (both 1:1,000) and mouse anti-human GAPDH primary antibody (1:4,000; both Abcam, Cambridge, UK) were added onto the membranes before incubation at 4˚C overnight. Then, the membrane was extensively washed with phosphate-buffered saline with Tween-20 (PBST) for 5 times of 5 min, and incubated with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:4,000; Abcam) at room temperature for 1 h. Subsequently, the membrane was washed with PBST for 5 times of 5 min before the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for imaging. We used Image lab v3.0 software (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to acquire and analyze imaging data. The relative expression of target proteins was expressed with the ratio against GAPDH.

Dual luciferase reporter assay. Wild-type (WT) and mutant seeding regions of miR-214 in 3'-UTR of DACT2 gene were chemically synthesized before adding Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids (0.5 μg) with WT or mutant 3'-UTR DNA sequences, which were transfected together with miR-214 mimics into HEK293T cells. Following incubation for 24 h, cells were processed using dual luciferase reporter assay kit according to the manufacturer's manual (Beyotime Institute of Biotechnology), and fluorescence intensity was determined by GloMax 20/20 luminometer (Promega, Fitchburg, WI, USA). Fluorescence values of each group of were measured using renilla fluorescence activity as internal reference.

Statistical analysis. All results were analyzed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA), and all data were shown as means ± SD. Comparison between groups was performed using group t-test. The results among multiple groups were compared using one-way analysis of variance followed by Dunnett's test as the post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-214 is elevated, but expression of Dact2 mRNA is decreased in gastric cancer tissues, being closely correlated with the invasion, metastasis, occurrence and development of gastric cancer. To search miRNA molecules that might participate in the regulation of Dact2 gene, Targetscan 7.1 website was used. Search of Dact2 on the website showed that miR-214 was likely to be involved in the regulation of Dact2 expression (Fig. 1). To further test the expression of miR-214 and Dact2 in gastric cancer, qRT-PCR was performed. The data showed that expression of miR-214

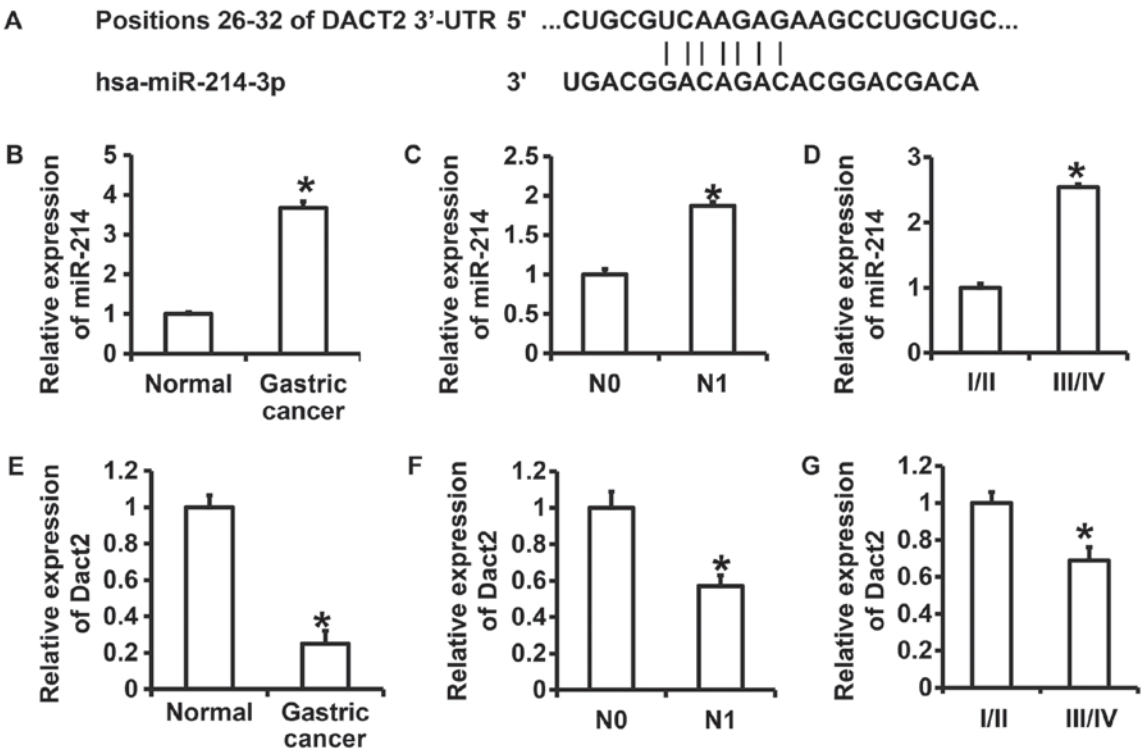

Figure 1. Prediction of interaction between miR-214 and Dact2, and the expression of miR-214 and Dact2 mRNA in gastric cancer tissues. (A) Bioinformatics prediction of miRNA molecule that regulates the expression of Dact2 gene using miRNA molecule online prediction software Targetscan 7.1 (www.targetscan.org/vert_71/). (B-D) Expression of miR-214 in (B) tumor-adjacent normal tissues and gastric cancer tissues (*P<0.05 compared with normal group), (C) patients without (N0)/with (N1) lymphatic metastasis (*P<0.05 compared with N0 group), and (D) patients at clinical stages I/II or III/IV (*P<0.05 compared with I/II group). (E-G) Expression of Dact2 mRNA in (E) tumor-adjacent normal tissues and gastric cancer tissues (*P<0.05 compared with normal group), (F) patients without (N0)/with (N1) lymphatic metastasis (*P<0.05 compared with N0 group), and (G) patients at clinical stages I/II or III/IV (*P<0.05 compared with I/II group). miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2.

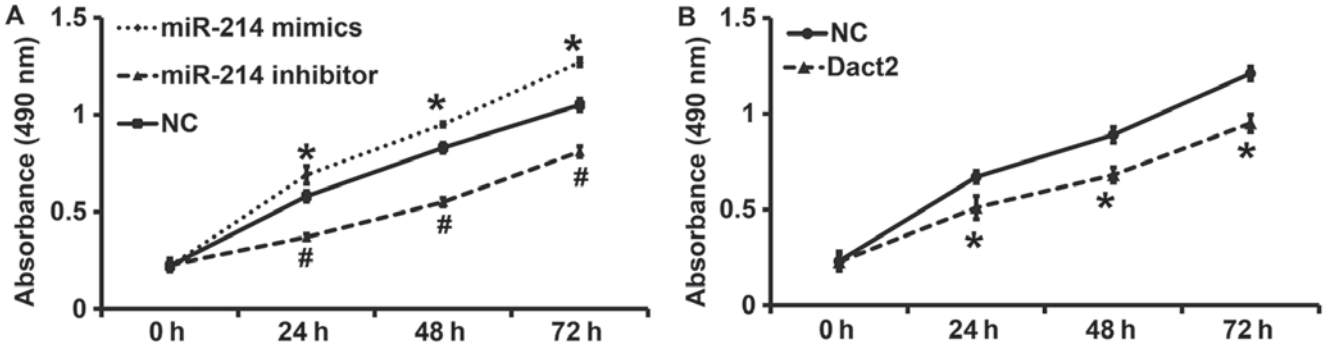

Figure 2. Effect of miR-214 and Dact2 gene on the proliferation of mixed gastric adenocarcinoma type MKN28 cells. CCK-8 assay was used to determine the proliferation of the cells. (A) Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC, miR-214 mimics or miR-214 inhibitor. *P<0.05 compared with NC group; #P<0.05 compared with miR-214 mimics group. (B) Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC or Dact2. *P<0.05 compared with NC group. NC, negative control; miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2; CCK-8, Cell-Counting kit.

in gastric cancer tissues was significantly higher than that in tumor-adjacent tissues (P<0.05) (Fig. 1B). In addition, miR-214 expression in N1 group was significantly higher than that in N0 group (P<0.05) (Fig. 1C). Of note, miR-214 expression in gastric cancer tissues from patients at stages III/IV was significantly higher than that from patients at stages I/II (P<0.05) (Fig. 1D). By contrast, the expression of Dact2 mRNA in gastric cancer tissues was significantly decreased than that in tumor-adjacent tissues (P<0.05) (Fig. 1E). The expression of Dact2 mRNA in gastric cancer tissues in N1 group was significantly lower than that in N0 group (P<0.05) (Fig. 1F). Moreover, the expression of Dact2 mRNA in gastric cancer tissues from patients at stages III/IV was significantly reduced than that from patients at stages I/II (P<0.05) (Fig. 1G). The results suggest that expression of miR-214 is elevated, but expression of Dact2 mRNA is decreased in gastric cancer tissues, being closely correlated with the invasion, metastasis, occurrence and development of gastric cancer.

miR-214 promotes, but Dact2 inhibits the proliferation of gastric cancer MKN28 cells in vitro. To examine the proliferation of mixed gastric adenocarcinoma type MKN28

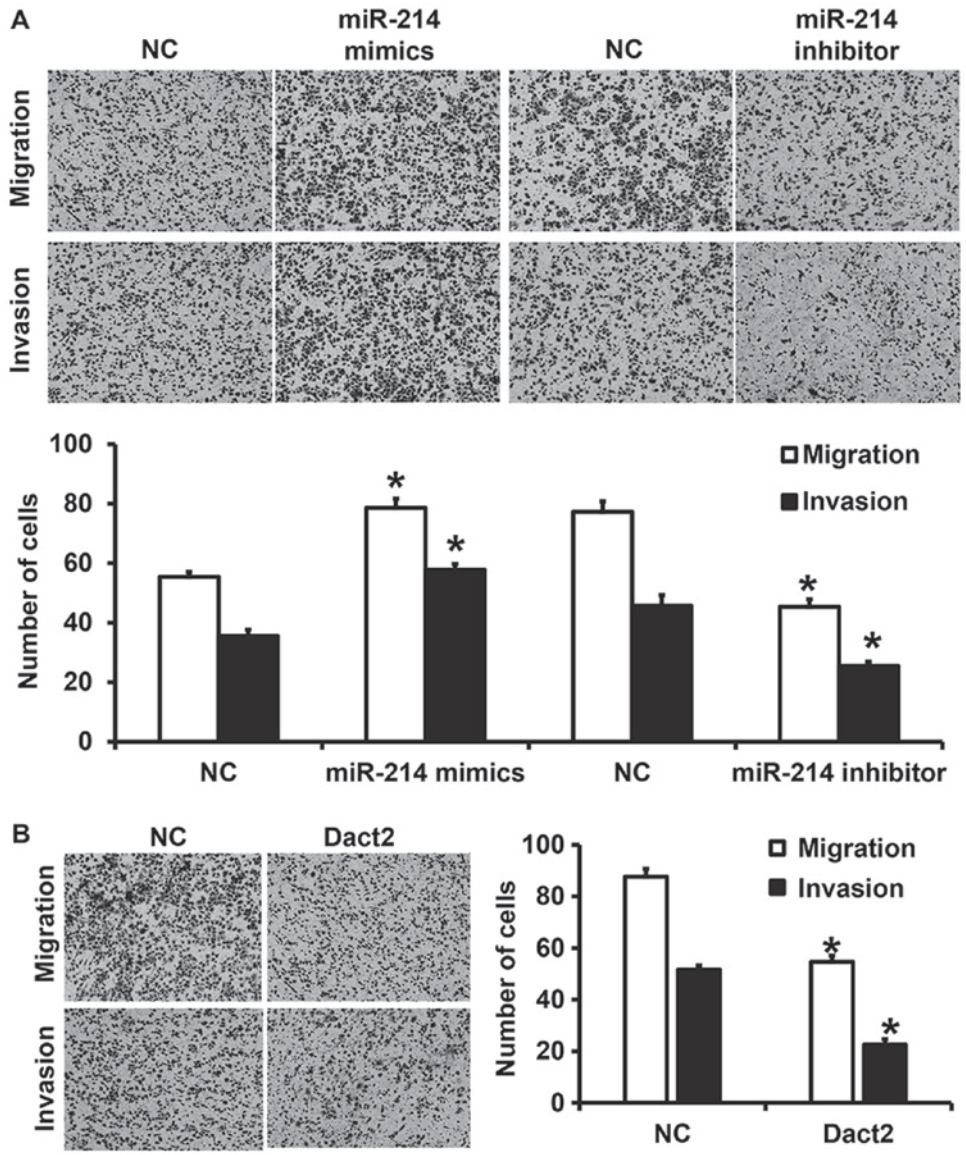

Figure 3. Effect of miR-214 and Dact2 on the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells. (A) Images and number of migrated and invasion cells in indicated groups. Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC, miR-214 mimics or miR-214 inhibitor. (B) Images and number of migrated and invasion cells in indicated groups. Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC or Dact2. Transwell assay was used to determine the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells (magnification, x100). *P<0.05 compared with NC group. NC, negative control; miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2.

cells, CCK-8 assay was carried out. The data showed that the absorbance of mixed gastric adenocarcinoma type MKN28 cells transfected with miR-214 mimics was significantly higher than that from cells in NC group at all time points (P<0.05), while the absorbance of the cells transfected with miR-214 inhibitor was significantly lower than that from cells in NC group at all time points (P<0.05) (Fig. 2A). By contrast, the absorbance of mixed gastric adenocarcinoma type MKN28 cells with overexpression of Dact2 gene was significantly lower than that of cells in NC group at all time points (P<0.05) (Fig. 2B). The results indicate that miR-214 promotes, but Dact2 inhibits the proliferation of gastric cancer MKN28 cells *in vitro*.

Downregulation of miR-214 expression or upregulation of Dact2 expression inhibits the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells. To investigate migration and invasion abilities of mixed gastric adenocarcinoma type MKN28 cells, Transwell assay was employed. The data showed that the numbers of cells in miR-214 mimics group that crossed chamber membrane in migration and invasion assays were increased than those in NC group (P<0.05). By contrast, cell counts in miR-214 inhibitor group that crossed chamber membrane in migration and invasion assays were lower than those in NC group (P<0.05). Similarly, cell count in miR-214 mimics group that crossed chamber membrane in invasion assay was reduced than that in miR-NC group (P<0.05) (Fig. 3A). Moreover, the numbers of cells in Dact2 group that crossed chamber membrane in migration and invasion assays were significantly reduced compared with those in NC group (P<0.05) (Fig. 3B). The results suggest that downregulation of miR-214 expression or upregulation of Dact2 expression inhibits the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells.

miR-214 regulates the expression of Dact2 protein and its downstream β-catenin protein in mixed gastric adenocarcinoma type MKN28 cells. To test Dact2 and β-catenin protein expression in mixed gastric adenocarcinoma type MKN28 cells, western blotting was performed. Quantification of western blots showed that Dact2 protein expression in miR-214 mimics group was significantly reduced than that in NC group (P<0.05), while β-catenin expression in miR-214 mimics group was significantly higher than that in NC group (P<0.05). By contrast, Dact2 protein expression in miR-214 inhibitor group was significantly higher than that in NC group (P<0.05), while β-catenin expression in miR-214 inhibitor group was significantly lower than that in NC group (P<0.05) (Fig. 4A). Moreover, transfection with Dact2 plasmid significantly elevated Dact2 expression in mixed gastric adenocarcinoma type MKN28 cells (P<0.05), but significantly reduced β-catenin expression (P<0.05) (Fig. 4B). The results indicate that miR-214 regulates the expression of Dact2 protein and its downstream β-catenin protein in mixed gastric adenocarcinoma type MKN28 cells.

Dact2 reverses the effect of miR-214 on the proliferation, migration and invasion of gastric cancer MKN28 cells. To test how the regulation of Dact2 by miR-214 affects biological functions of mixed gastric adenocarcinoma type MKN28 cells, we silenced and rescued the expression of miR-214. Western blotting showed that Dact2 expression in miR-214 inhibitor group was significantly higher than that in NC group (P<0.05), while that in rescue group was significantly lower than that in miR-214 inhibitor group (P<0.05) (Fig. 5A). CCK-8 assay showed that the absorbance of cells in miR-214 inhibitor group was significantly reduced than that in NC group at all time points (P<0.05), while that in rescue group was significantly higher than that in miR-214 inhibitor group at all time points (P<0.05), reaching a level similar to NC group (Fig. 5B). Moreover, Transwell assay showed that the numbers of cells in miR-214 inhibitor group that crossed chamber membrane in migration and invasion assays were significantly lower than those in NC group, respectively (P<0.05), while those in rescue group were significantly higher than those in miR-214 inhibitor group (P<0.05) (Fig. 5C). The results suggest that Dact2 reverses the effect of miR-214 on the proliferation, migration and invasion of mixed gastric adenocarcinoma type MKN28 cells.

miR-214 can bind with the 3'-UTR seeding region of Dact2 mRNA to regulate its expression. To identify the interaction between miR-214 and the 3'-UTR of Dact2 mRNA, dual luciferase reporter assay was performed. The fluorescence value of cells co-transfected with miR-214 mimics and pMIR-REPORT-WT luciferase reporter plasmids was significantly lower than that in NC group (P<0.05). By contrast, the fluorescence value of cells co-transfected with miR-214 mimics and pMIR-REPORT-mutant luciferase reporter plasmids was not significantly different from that in NC group (P>0.05) (Fig. 6). The result indicates that miR-214 can bind with the 3'-UTR seeding region of Dact2 mRNA to regulate its expression.

Discussion

Gastric cancer is a systemic disease, and its recurrence and metastasis are the major causes of poor prognosis (4). Similar

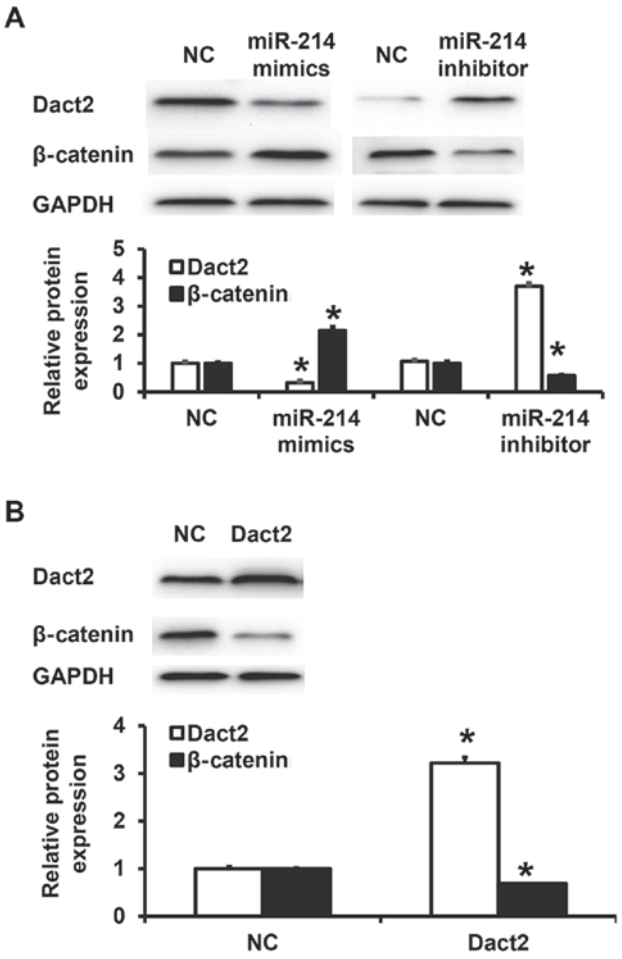

Figure 4. Effect of miR-214 on the expression of Dact2 and its downstream protein β-catenin. (A) Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC, miR-214 mimics or miR-214 inhibitor before determination of Dact2 and β-catenin protein expression by western blotting. (B) Mixed gastric adenocarcinoma type MKN28 cells were transfected with NC or Dact2 before determination of Dact2 and β-catenin protein expression by western blotting. *P<0.05 compared with NC group. NC, negative control; miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2.

to other solid tumors, the proliferation and metastasis of gastric cancer cells are regulated by multiple genes and multiple factors (22,23). miRNA has powerful post-transcriptional regulation function and a wide application prospect in tumor therapy. It is reported that many miRNA molecules are expressed abnormally in gastric cancer cells and play important roles in the occurrence and development of gastric cancer (24).

Wnt/β-catenin signaling pathway is abnormally activated in many tumors, and promotes tumor formation, maintenance, invasion, metastasis and drug resistance (25). As an important negative regulator of Wnt/β-catenin signaling pathway, Dact2 has become a focus among researchers (26). The deletion of Dact2 leads to the activation of Wnt/β-catenin signaling pathway, and facilitates the occurrence and development of colon cancer, esophageal cancer and liver cancer. Studies show that the deletion of Dact2 is related to the methylation of promoter region. For example, methylation of the Dact2 gene promoter leads to the silencing of Dact2 gene, and promotes epithelial mesenchymal transition and cytoskeletal rearrangement in

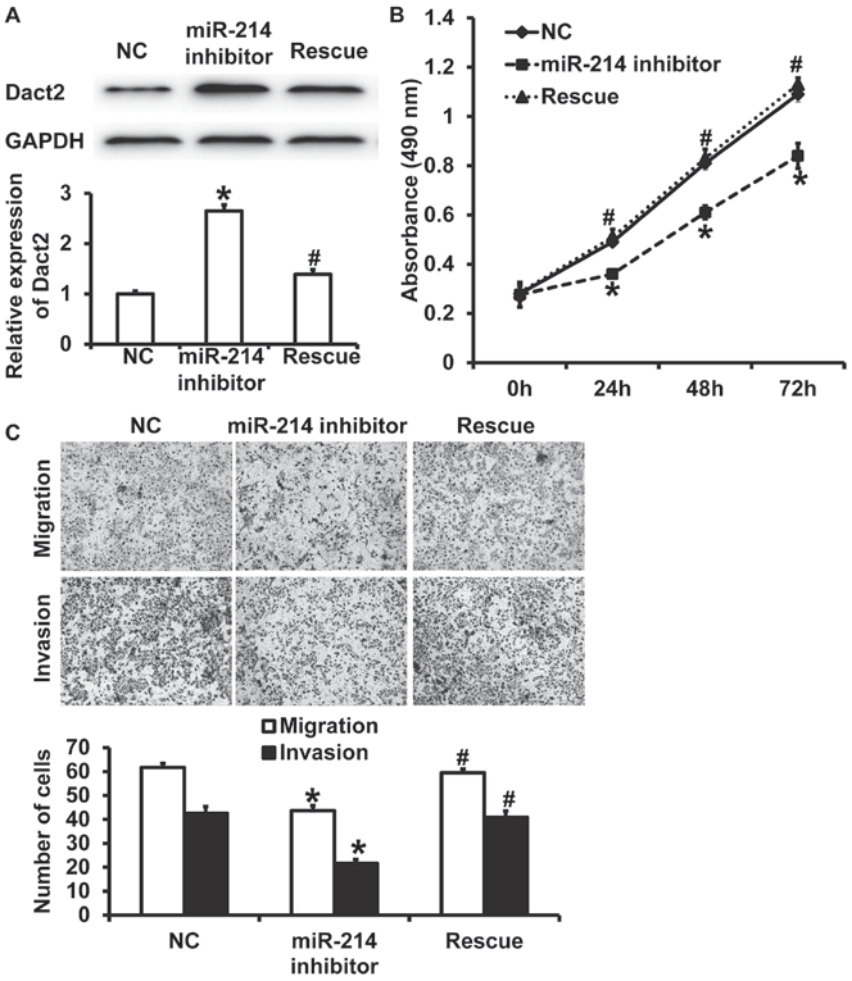

Figure 5. Effect of Dact2 expression regulated by miR-214 on the proliferation, migration and invasion of mixed gastric adenocarcinoma type MKN28 cells. (A) Effect of inhibition and rescue of miR-214 expression on the expression of Dact2 protein determined by western blotting. (B) Effect of inhibition and rescue of miR-214 expression on the proliferation of mixed gastric adenocarcinoma type MKN28 cells determined by CCK-8 assay. (C) Effect of inhibition and rescue of miR-214 expression on the migration and invasion of mixed gastric adenocarcinoma type MKN28 cells determined by Transwell assay (magnification, x100). Statistical comparisons among the NC, miR-214 inhibitor and Rescue groups were performed using ANOVA and Dunnett's test as a post-hoc test. Statistical analysis showed no significant difference between Rescue and NC groups, but miR-214 was significantly different from Rescue and NC groups. *P<0.05 compared with NC group; #P<0.05 compared with miR-214 inhibitor group. NC, negative control; miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2; CCK-8, Cell-Counting kit.

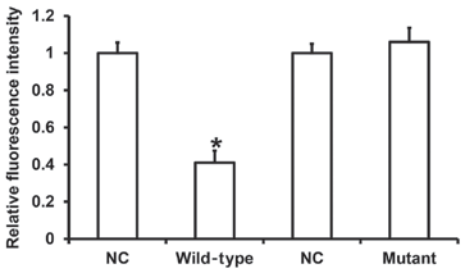

Figure 6. Identification of interaction between miR-214 and Dact2 mRNA using dual luciferase reporter assay. WT and mutant seeding regions of miR-214 in 3'-UTR of DACT2 gene were chemically synthesized before adding Spe-1 and HindIII restriction sites, and then cloned into pMIR-REPORT luciferase reporter plasmids (0.5 μg) with WT or mutant 3'-UTR DNA sequences, which were transfected together with miR-214 mimics into HEK293T cells. Following incubation for 24 h, cells were processed using dual luciferase reporter assay kit according to the manufacturer's manual, and fluorescence intensity was determined by GloMax 20/20 luminometer. Fluorescence values of each group of were measured using renilla fluorescence activity as internal reference. *P<0.05 compared with respective NC group. NC, negative control; WT, wild-type; miRNA, microRNA; Dact2, dapper, antagonist of β-catenin 2; UTR, untranslated region.

breast cancer (27). Moreover, aberrant methylation of Dact2 promoter in squamous cell carcinoma of esophagus is the key reason for silenced expression of Dact2, and this ultimately promotes the invasion and metastasis of squamous cell carcinoma (28). However, promoter methylation does not necessarily exist in tumor tissues with downregulated Dact2 expression, suggesting that Dact2 has other regulatory mechanisms.

miRNA molecules are key regulators of mRNA at posttranscriptional levels, and they are almost involved in all physiological and pathological activities of the body. Studies have shown that a variety of miRNA molecules have been involved in the regulation of Wnt/β-catenin signaling pathway. For example, Liu *et al* discover that miR-155 promotes the proliferation and metastasis of SW-480 cells by regulating Wnt/β-catenin signaling pathway (29). Wei *et al* find that miR-638 inhibits the metastasis of cervical cancer cells by regulating Wnt/β-catenin signaling pathway (30). The discovery of these miRNA molecules

provides new targets for the regulation of Wnt/β-catenin signaling pathway. In the present study, we use bioinformatics to predict that miR-214 may regulate Dact2 gene. Using qRT-PCR, we identified upregulated expression of miR-214 in gastric cancer tissues, which is positively related with clinical staging and lymphatic metastasis. Moreover, expression of Dact2 gene is downregulated, and negatively correlated with clinical staging and lymphatic metastasis. Cellular functional experiments show that miR-214, as an oncogene, promotes the proliferation, migration and invasion of gastric cancer cells. By contrast, Dact2 acts as a tumor-suppressor gene that inhibits the proliferation, migration and invasion of gastric cancer cells. These results demonstrate that the function and expression of miR-214 are reversely related with Dact2, suggesting that miR-214 may exert its biological functions via Dact2. Western blotting shows that miR-214 regulates the expression of Dact2. Furthermore, rescue experiments demonstrate that Dact2 inhibits the tumor-promoting effect of miR-214. Indeed, dual luciferase reporter assay shows that miR-214 directly binds with the 3'-UTR of Dact2 mRNA.

In conclusion, miR-214 downregulates the expression of Dact2 gene, activates Wnt/β-catenin signaling pathway, and promotes the proliferation, migration and invasion of gastric cancer. Therefore, miR-214 is a potential molecular therapeutic target and biomarker for gastric cancer. In our future studies, we will investigate the correlation between the expression of miR-214 and Dact2 in tissues, and test the function and mechanism of action of miR-214 at animal level.

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Availability of data and materials

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

Authors' contributions

LZ designed the study. WF, YF and SG were responsible for performing experiments. LZ and WF analyzed the data. All authors collaborated to interpret results and develop the manuscript. The final version of the manuscript has been read and approved by all authors.

Ethics approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Harbin Medical University. Written informed consent was obtained from all patients or their families.

Patient consent for publication

Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

Competing interests

The authors declare that they have no competing interests.

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