P-Akt/miR-200 signaling regulates epithelial-mesenchymal transition, migration and invasion in circulating gastric tumor cells

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Abstract. Both circulating tumor cells (CTCs) and epithelial-mesenchymal transition (EMT) play an important role in invasion, migration and chemoresistant in tumor development. This study aimed to detect whether EMT occurred in human gastric CTCs and to explore the mechanism of EMT in human gastric CTCs. We analysed epithelial markers (pan-CK, E-cadherin), mesenchymal markers (N-cadherin, vimentin) EMT related miR-200s, and Akt in gastric CTCs. The impact of miR-200s on EMT, migration and invasion in CTCs was tested. We found that epithelial markers pan-CK, E-cadherin were decreased, and mesenchymal markers N-cadherin, vimentin were overexpressed in gastric CTCs. Expression of EMT related transcriptors, snail1, zeb1, twist1, were reversely correlated with miR-200s, and were positively correlated with phospho-Akt. Upregulated of miR-200s downregulated twist1 and zeb1 mRNA expression, and resulted in the supression of EMT, and impaired migration and invasion in gastric CTCs. Inhibition of p-Akt led to upregulation of miR-200s. In conclusion, gastric CTCs exhibited remarkable EMT process, and p-Akt/miR-200s signaling regulates EMT, migration and invasion in gastric CTCs.

Introduction

According to statistics of 2012 International Agency for Research on Cancer (IARC), almost one million new cases of gastric cancer were estimated to have occurred in 2012 (952,000 cases, 6.8% of the total), making it the fifth most common malignancy in the world, after cancers of the lung, breast, colorectum and prostate. More than 70% of cases (677,000 cases) occur in developing countries (456,000 in men, 221,000 in women), and half the world total occurs in Eastern Asia (mainly in China). Gastric cancer is the third leading cause of cancer death in both genders worldwide (723,000 deaths, 8.8% of the total), and the third leading cause of cancer death in both males and females in China (1). The poor prognosis of gastric cancer is due to its metastasis and relapse. Metastasis, as a result of dissemination and growth of cancer cells, represents the most common cause of death in cancer patients. Therefore, it is necessary to characterize the molecular mechanism of gastric cancer metastasis.

Circulating tumor cells (CTCs) are cells that leave the primary tumor and circulate in the periphery blood. CTCs are both prognostic and predictive marker for cancer patients (2-4). Previous studies also demonstrated that CTCs could help clinicians learn more about tumor biological behavior, such as, chemo-sensitivity or resistance and metastatic ability, and CTCs could provide for screening for adjuvant therapy patients, and tumor pharmacokinetics, and provide new targets for treatment (5). It is reported that CTCs participated in the initial process of metastasis (6). Thus, CTCs may have a potential utility as less invasive than standard biopsies to further understand the mechanism of metastasis.

Epithelial-mesenchymal transition (EMT) plays an important role in tumor cell invasion and metastasis (7). EMT is a complex process that refers to the transformation of epithelial cells to mesenchymal cells, in which the polarity of epithelial cells is lost, accompanied with enhanced migration and invasion (8). The characteristics of EMT is the loss of expression of epithelial cell markers (E-cadherin) and overexpression of mesenchymal cell markers (e.g., α-smooth muscle actin protein, α-SMA, N-cadherin, vimentin) (8-10). Recently, Yu *et al* reported that circulating breast tumor cells exhibited dynamic changes in epithelial and mesenchymal characteristics during treatment of breast cancer patients (11). In addition, miR-200b has been reported to inhibit EMT in prostate cancer cells (12). Virtakoivu *et al* found that inhibited Akt2 could

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Abbreviations: CTCs, circulating tumor cells; EMT, epithelial-mesenchymal transition; miR, microRNA

Key words: circulating tumor cells, epithelial-mesenchymal transition, miR-200s, p-Akt

induce overexpression of miR-200s and then regulated invasion and migration of prostate cancer cells (13). Our previous study confirmed that gastric CTCs had stronger capacity of migration, invasion, metastasis, and radiation-resistance than human gastric cancer cell lines SGC-7901 and MKN-45. Thus, this study aimed to detect whether EMT occurred in gastric CTCs and participated in the process of human gastric cancer metastasis, and then to find the mechanism of EMT in human gastric CTCs.

Materials and methods

Cell lines. GES, SGC-7901, MKN-45 cell lines were purchased from Chinese Academy of Medical Sciences Cancer Cell Bank (Beijing, China). The human gastric circulating tumor cells (CTC-105, CTC-141, CTC-1, CTC-12) used in this study were previously established by $CD44^+/CD45^-$ isolation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Beijing, USA). All of the cell lines were maintained in a humidified atmosphere containing 5% CO_2.

Western blot analysis. Protein extracts were resolved by 12% SDS-PAGE electrophoresis (Bio-Rad, USA), and transferred to PVDF membranes (Millipore, MA, USA), and probed with antibodies against human N-cadherin (1:4,000, Epitomics, USA), E-cadherin (1:1,000, Abcam, USA), vimentin (1:4,000, Epitomics), Akt (1:1,000, CST, USA), phospho-Akt (473) (1:4,000, Epitomics), anti-zeb1 antibody (1:1,000, Abcam) or GAPDH (1:1,000, Sigma, USA). Fluorescence-conjugated anti-mouse or rabbit IgG (1:10,000, Sigma) were used as the secondary antibodies, and the antigen-antibody reactions were visualized using LI-COR Odyssey Infrared Imaging System (USA). Triple tests were replicated.

Triple staining of immunofluorescence analysis. Cells were fixed with 4% paraformaldehyde for 20 min and blocked in 1X PBS (pH 7.4) solution with 1% BSA. The anti-N-cadherin (1:400, Epitomics), anti-E-cadherin (1:300, Abcam), anti-vimentin (1:400, Epitomics), or anti-pan-CK (1:300, Abcam), were added and incubated overnight at 4˚C in a humidified box. After washing, the fluorescent secondary antibody (Epitomics) was added at a dilution of 1:400 and incubated for 1.5 h. The cells were then washed three times with PBS, and counterstained with DAPI (Sigma) for 2 min. Fluorescence was analyzed using a fluorescent microscope (Zeiss, Germany). Triple tests were replicated.

Quantitative real-time PCR. Total RNA was isolated with the TRIzol reagent (Invitrogen, USA) from cells according to the manufacturer's instructions. Single-strand cDNA was synthesized from 1 μg of total RNA by reverse transcription according to the manufacturer's instructions (Takara, China). Single-strand cDNA for miRNA was synthesized by reverse transcription using miRNA cDNA kit according to the manufacturer's instructions (CW Biotech, China). Quantitative real-time PCR was used to measure the mRNA levels of E-cadherin, N-cadherin, vimentin, snail1, twist1, zeb1 and miRNA levels of miR-200a, b and c in gastric circulating tumor cells and gastric cancer cell lines. Quantitative PCR was performed on Bio-Rad CFX manager (USA). Amplification was carried out in a 20-μl volume in triplicate for 40 cycles and the product was detected using SYBR Green fluorochrome. The geometric average Ct value was used to calculate relative expression of the above genes using the method $2^{-\Delta\Delta CT}$. U6 and GADPH were used as endogenous control. Triple tests were replicated. The primers were synthesized by Genwiz Co. (Suzhou, China). The primers are listed in Table I).

Table I. Primer sequences of related genes.

Gene	Primers
E-cadherin	Forward: 5'-CGAGAGCTACACGTTCACGG-3'
	Reverse: 5'-GGGTGTCGAGGGAAAAATAGG-3'
N-cadherin	Forward: 5'-AGCCAACCTTAACTGAGGAGT-3'
	Reverse: 5'-GGCAAGTTGATTGGAGGGATG-3'
Vimentin	Forward: 5'-GACGCCATCAACACCGAGTT-3'
	Reverse: 5'-CTTTGTCGTTGGTTAGCTGGT-3'
snail1	Forward: 5'-TCGGAAGCCTAACTACAGCGA-3'
	Reverse: 5'-AGATGAGCATTGGCAGCGAG-3'
twist1	Forward: 5'-GTCCGCAGTCTTACGAGGAG-3'
	Reverse: 5'-GCTTGAGGGTCTGAATCTTGCT-3'
zeb1	Forward: 5'-TTACACCTTTGCATACAGAACCC-3'
	Reverse: 5'-TTTACGATTACACCCAGACTGC-3'
GADPH	Forward: 5'-CTGCACCACCAACTGCTTAG-3'
	Reverse: 5'-TGAAGTCAGAGGAGACCACC-3'
U6	miRNA qPCR Primer Set (RiboBio, MQP-0201)
miR-200a	miRNA qPCR Primer Set (Tiangen, CD201-0022)
miR-200b	miRNA qPCR Primer Set (Tiangen, CD201-0023)
miR-200c	miRNA qPCR Primer Set (Tiangen, CD201-0024)

Migration and invasion assay. Cell migration and invasion assays were performed as follows. For invasion assay, $1x10^4$ cells were seeded on an 8-μm-pore size Transwell insert (BD, USA) coated with extracellular matrix (ECM) (1:4) (BD), while in the migration assay ECM was not used. After 48 h of incubation at 37˚C and 5% CO_2, cells adherent to the upper surface of the filter were removed. Cells were stained with hematoxylin-eosin, and the number of cells on the bottom were counted under a microscope. Triple tests were replicated.

Hsa-miR-200s mimic transfection. Cells ($20x10^4$) were seeded in 6-well plates and grown to 50-60% confluence. Human hsa-miR-200b and c (RiboBio, Guangzhou, China) or its negative control (RiboBio, Guangzhou, China) was directly transfected into circulating gastric tumor cells in free of serum Opti-MEM (Invitrogen) at a final concentration of 50 nmol, according to the manufacturer's protocol.

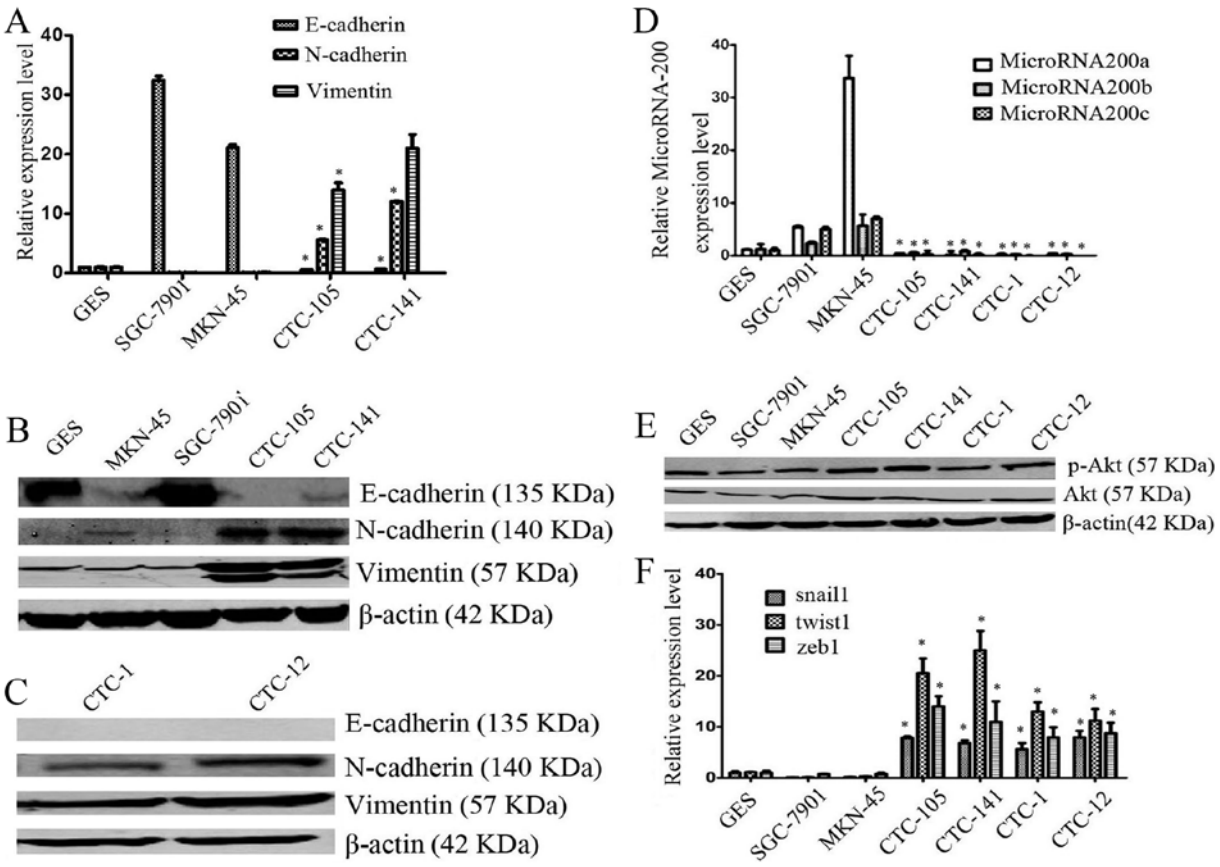

Figure 1. EMT related markers were detected in gastric CTCs and gastric cancer cell lines. (A) EMT related markers were detected in gastric CTCs and gastric cancer cell lines by quantitative real-time PCR, relative expression of the genes was calculated using the method $2^{-\Delta\Delta CT}$. (B) EMT related markers were tested in gastric CTCs and gastric cancer cell lines by western blotting. (C) Proteins were detected in two gastric CTCs by western blotting. (D) MicroRNA-200s were detected in gastric CTCs and gastric cancer cell lines by quantitative real-time PCR. (E) Total Akt and phosphor-Akt protein levels were detected in gastric CTCs and gastric cancer cell lines by western blotting. (F) EMT related transcription factors were detected in gastric CTCs and gastric cancer cell lines by quantitative real-time PCR. GES, the normal gastric mucosa epithelial cells, were used as control for quantification. Data are presented as means ± SD from three independent experiments, one of three similar experiments is shown, *P<0.05, vs SGC-7901 and MKN-45.

Statistical analysis. The quantitative data are presented as mean values ± SD from three independent experiments. One-way analysis of variance (ANOVA) was used to analyze differences among groups. In addition, the LSD multiple comparison test was used to identify differences among means of two different groups. Test level of α was 0.05, P-values <0.05 were considered statistically significant.

Results

Gastric CTCs exhibit remarkable EMT process. To examine the differences of EMT phenotype between gastric circulating tumor cells and gastric cancer cell lines SGC-7901 and MKN-45, the mRNA and protein levels of EMT markers were measured. The relative mRNA levels of mesenchymal markers N-cadherin, and vimentin were significantly overexpressed in circulating gastric tumor cells, compared with gastric cancer cell lines (*P<0.05 for all comparation, Fig. 1A), whereas the relative mRNA level of E-cadherin was significantly decreased (*P<0.05 for all comparisons, Fig. 1A), as assessed by real-time PCR assay. The results from western blot analysis demonstrated that the relative protein levels of N-cadherin, and vimentin were overexpressed (Fig. 1B and C), whereas expression of E-cadherin was low in the circulating gastric tumor cells, as compared with gastric cancer cell lines, SGC-7901and MKN-45 (Fig. 1B and C). These results indicated that the expression of mesenchymal biomarkers was elevated, while expression of epithelial biomarkers was decreased, further studies of triple staining immunofluorescence suggested that, compared with gastric cancer cell lines, CTCs highly expressed the mesenchymal biomarkers vimentin and N-cadherin, but lowly or weakly expressed epithelial biomarkers E-cadherin and pan-CK (Figs. 2 and 3).

Expression of EMT related transcriptors was reversely correlated with miR-200s and was positively correlated with Akt kinases activation in gastric CTCs. To investigate the role of miR-200s, a family of EMT-associated miRNAs, in human gastric CTCs, the relative expression of miR-200s were examined by quantitative real-time PCR assay. As expected, the relative expression of miR-200a, b and c were all significantly decreased in human gastric circulating tumor cells as compared with SGC-7901 and MKN-45 cells (P<0.05 for all comparisons, Fig. 1D), which may suggested that miR-200s were involved in the process of EMT. To evaluate whether PI3K and Akt kinases signaling pathway is activated to participate in the process of EMT in gastric CTCs western blotting was performed to detect expression of both Akt and p-Akt

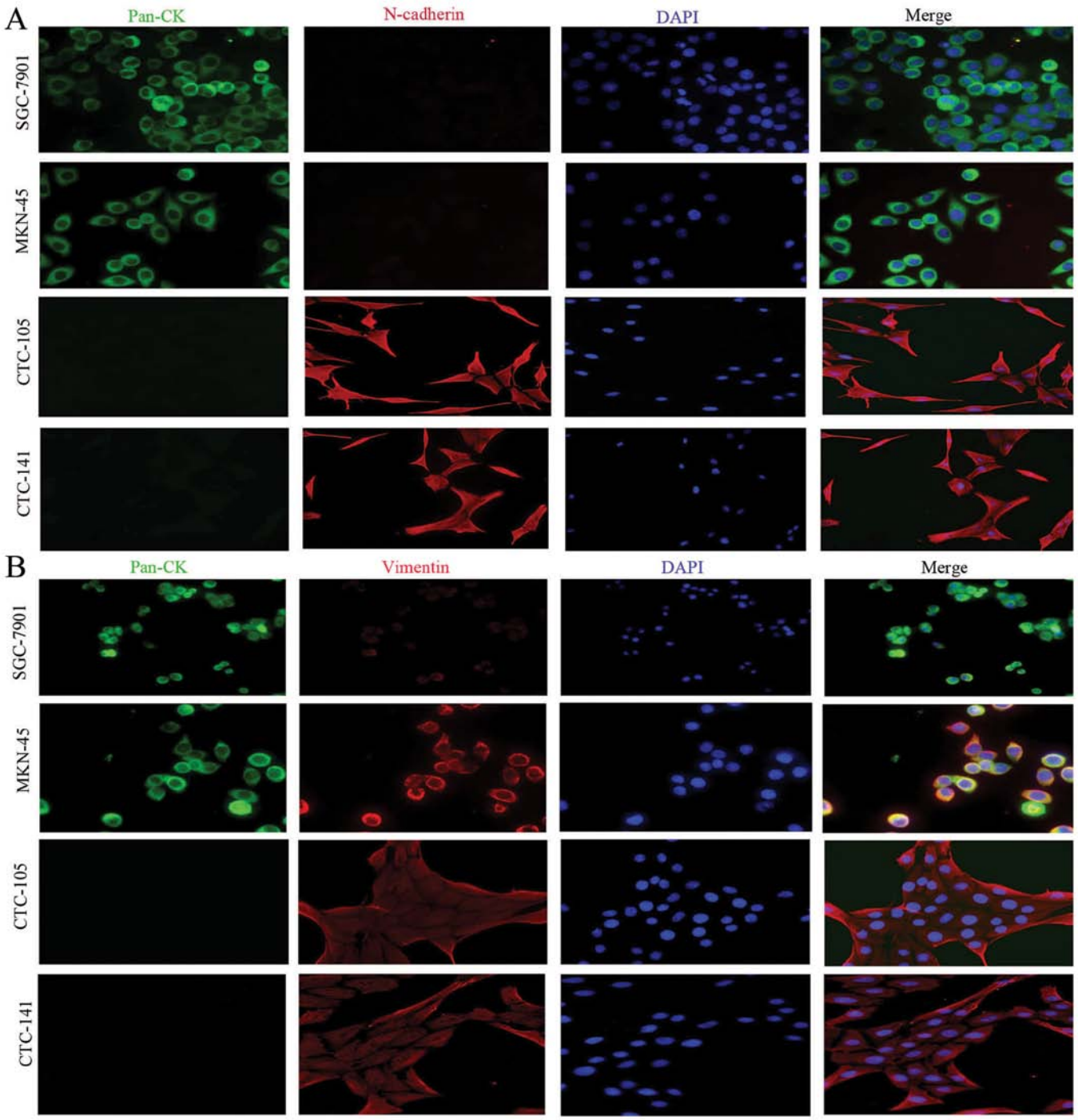

Figure 2. Epithelial marker pan-CK and mesenchymal marker vimentin and N-cadherin were detected in gastric CTCs and gastric cancer cell lines by triple immunofluorescent staining (x40). (A) pan-CK (green)/N-cadherin (in red)/DAPI (in blue) were co-stained in gastric CTCs and gastric cancer cell lines. (B) pan-CK (green)/vimentin (red)/DAPI (blue) were co-stained in gastric CTCs and gastric cancer cell lines. One of three similar experiments is shown.

(s473). It was observed that expression of both Akt and p-Akt (s473), especially p-Akt (s473), were activated in gastric CTCs (Fig. 1E). Expression of EMT-related transcription factor snail1, twist1, zeb1 was significantly overexpressed in gastric CTCs, compared with gastric cancer cell lines ($P<0.05$ for all comparation, Fig. 1F). Based on the above, expression of EMT related transcriptors was reversely correlated with miR-200s and was positively correlated with Akt kinase activation in gastric CTCs.

miR-200b and c promoted E-cadherin expression and decreased twist1, zeb1 expression in gastric CTCs. In order to investigate the impact of miR-200b and c on human gastric CTCs, human hsa-miR-200b and c or negative control miRNA were transfected into human gastric CTCs. After transfection, triple staining immunofluorescence assays were performed. Ectopic expression of miR-200b and c increased E-cadherin expression in gastric CTCs (Fig. 4). In addition, compared with negative control miRNA, ectopic expression

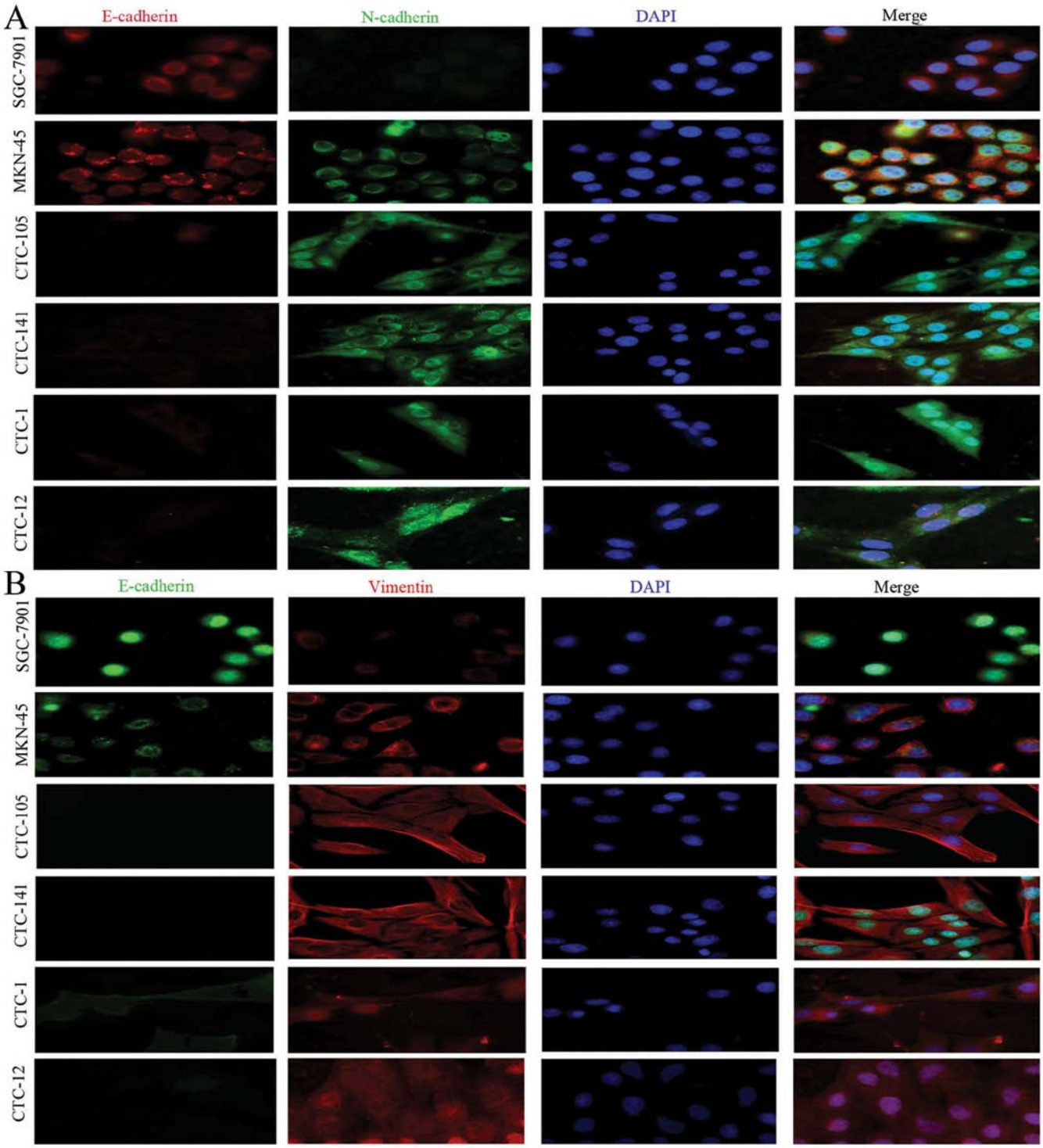

Figure 3. Triple immunofluorescent staining of epithelial marker E-cadherin and mesenchymal marker vimentin, N-cadherin in gastric CTCs and gastric cancer cell lines (x63). (A) E-cadherin (red)/N-cadherin (green)/DAPI (blue) were co-stained in gastric CTCs and gastric cancer cell lines. (B) E-cadherin (green)/vimentin (red)/DAPI (blue) were co-stained in gastric CTCs and gastric cancer cell lines. One of three similar experiments is shown.

of miR-200b or c decreased mRNA expression of snail1, twist1, zeb1 by 7.6-15.6, 2-3 and 1.58-2.3 times, respectively, in CTC-105 cells (P-values were all <0.05, Fig. 5A). mRNA expression of twist1, zeb1 in CTC-141 was downregulated by 1.5-2 and 1.3-1.5 times, respectively, after transfection of miR-200b or c (P-values were all <0.05, Fig. 5A), while snail1 expression tended to increase, no significant difference was observed (P>0.05, Fig. 5A). Consistent with the results of real-time PCR, western blotting demonstrated that ectopic expression of miR-200b and c decreased the expression of zeb1 protein (Fig. 5B). Overall, miR-200b and c promoted E-cadherin expression and decreased twist1, zeb1 expression in gastric CTCs.

miR-200b and c inhibited migration and invasion in gastric CTCs. miR-200b and c are known to play key roles in the

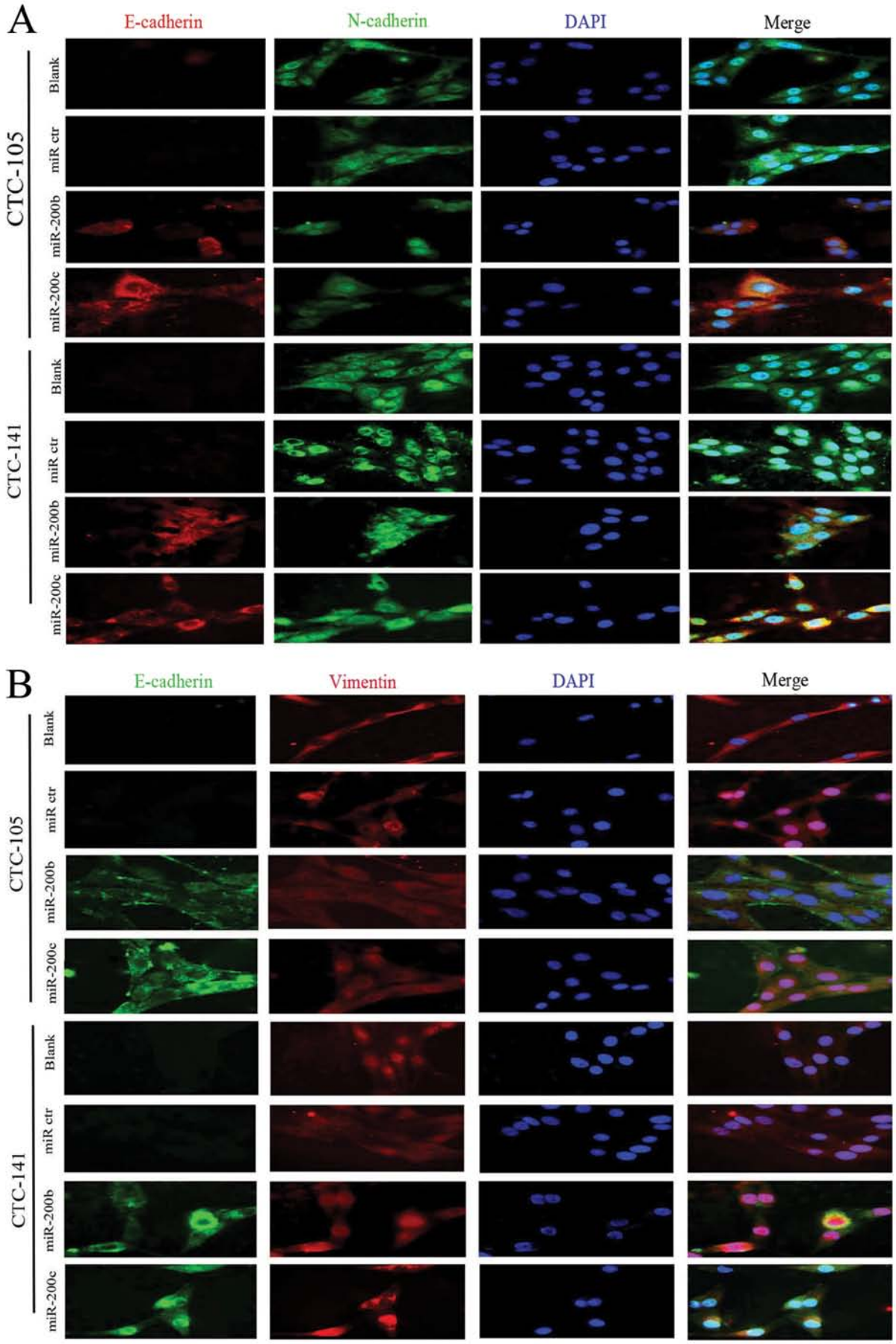

Figure 4. E-cadherin, N-cadherin and vimentin were stained after transfection of miR-200s into gastric CTCs by triple immunofluorescent staining (x63). (A) E-cadherin (red)/N-cadherin (green)/DAPI (blue) were co-stained in gastric CTCs. (B) E-cadherin (green)/vimentin (red)/DAPI (blue) were co-stained in gastric CTCs. One of three similar experiments was shown. Blank, CTC was not transfected; miR ctr, CTC trasfected with negative control miRNA.

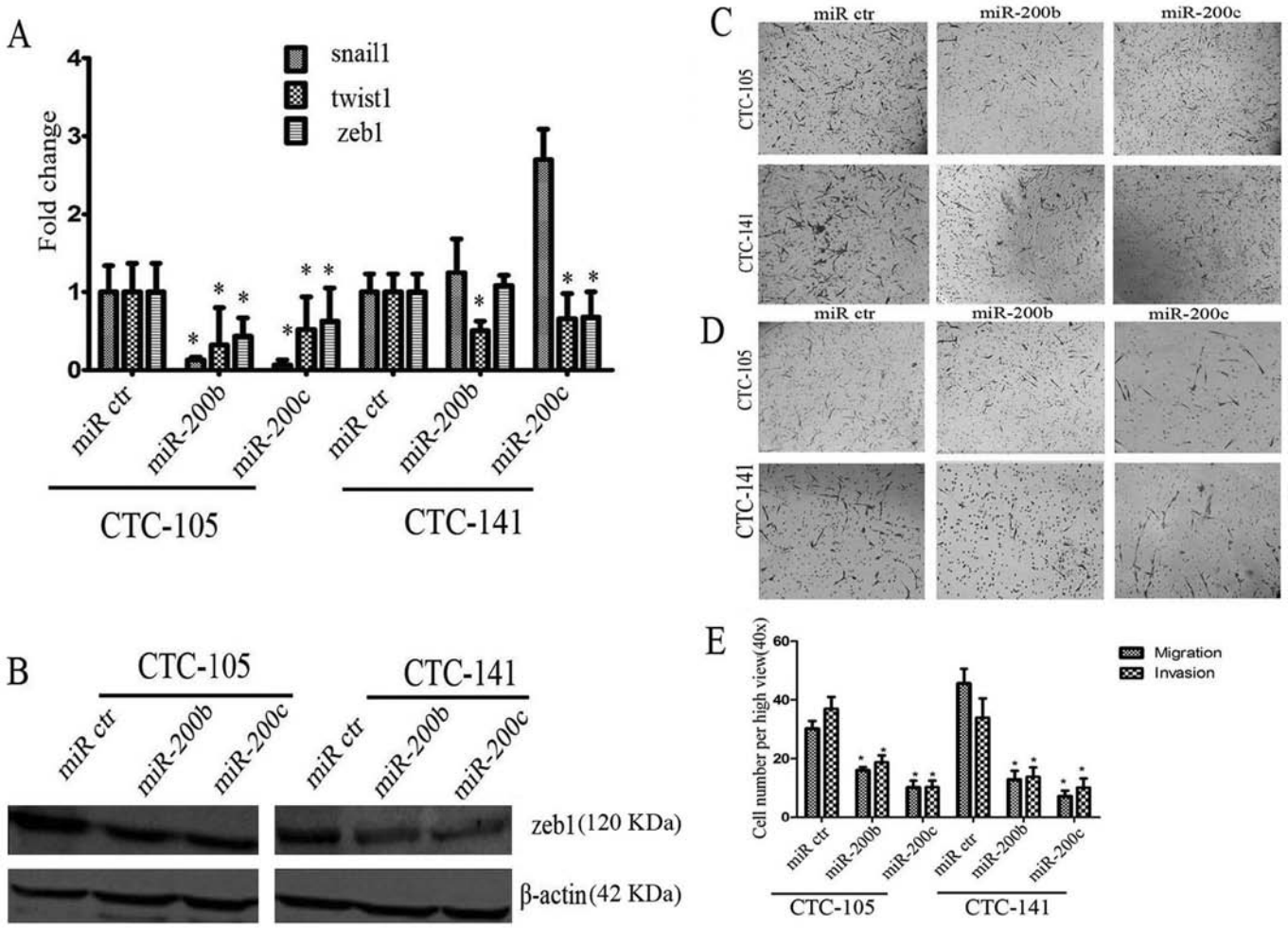

Figure 5. EMT related transcriptors and property of migration and invasion of CTCs were calculated after transfection of miR-200s. (A) Fold change of twist1, snail1, zeb1 mRNA levels were calculated after transfection of miR-200s into gastric CTCs by quantitative real-time PCR, relative expression of the genes was calculated using the method $2^{-\Delta\Delta CT}$ (*P<0.05, vs miR ctr). (B) Zeb1 protein level was determined by western blotting after transfection of miR-200s into gastric CTCs. (C) Representative results of transwell migration assays of CTCs scanned through a microscope (x10 original magnification). (D) Representative results of transwell invasion assays of CTCs scanned through microscope (x10 original magnification). (E) Number of cells per high view that migrated through the transwell membrane. Results are presented as means of 15 high power views (x40 original magnification). A representative experiment in triplicate, along with standard errors is shown. One-way analysis of variance (ANOVA) was used to analyze differences among groups and the LSD multiple comparison test was used to identify differences among different groups (*P<0.05, vs miR ctr). miR ctr, CTCs trasfected with negative control miRNA.

regulation of cell migration and invasion. To test whether the cell migration and invasion potential of gastric CTCs transfected with miR-200b or c were inhibited, transwell assay was performed. As shown in Fig. 5C-E, compared with negative control miRNA, ectopic expression of miR-200b or c inhibited migration and invasion potential (P<0.05 for all comparisons, Fig. 5E). Based on the above, miR-200s play a negative role in invasion and metastasis of human gastric CTCs. These results indicated that miR-200b and c inhibited migration and invasion potential in gastric CTCs.

Inhibition of p-Akt increased expression of MicroRNA-200s in gastric CTCs. Virtakoivu *et al* found that inhibited Akt2 could induce overexpression of miR-200s and then regulated invasion and migration of prostate cancer cells (13). To detect the Akt kinase activation on the expression of miR-200s, PI3K inhibitor 10 μM LY294002 was added to CTCs and then expression of miR-200s were detected by real-time PCR. As shown in Fig. 6, the PI3K inhibitor LY294002 inhibited phosphorylation of AKt, and increased the expression of miR-200a, b and c both in CTC-105 and CTC-141 cells. This effect suggests that the function of miR-200s in the gastric CTCs is likely, at least in part, regulated by p-AKt signaling pathway.

Discussion

Epithelial-mesenchymal (EM) transition (EMT) is a process where cells lose their epithelial properties and obtain mesenchymal properties. EMT has the ability to enhance tumor cell invasion and metastasis (7). Usually in the process, suppressed expression of E-cadherin can induce increased expression of N-cadherin, which is closely related to tumor invasion. Except for invasion, EMT was also reported in the process of drug resistance through multisignal transduction. For example, Ren *et al* found that inhibition of ZEB1 reverses EMT and chemoresistance in docetaxel-resistant human lung adenocarcinoma cell line (14), Rosano *et al* found that activated endothelin A

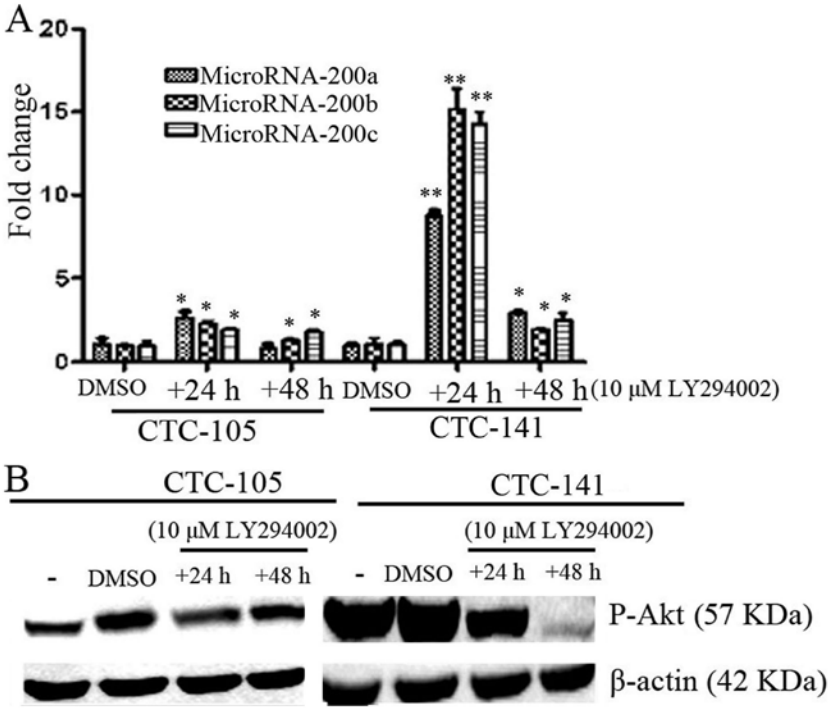

Figure 6. Effect of p-Akt on expression of miR-200s. (A) miR-200s were calculated after addition of PI3K inhibitor LY294002 24 and 48 h by quantitative real-time PCR. Fold changes were calculated using the method $2^{-\Delta\Delta CT}$. (B) p-Akt protein level was detected in CTCs after addition of PI3K inhibitor LY294002 by western blotting. DMSO, as control. Data are presented as means ± SD from three independent experiments, one of three similar experiments is shown, ($^{*}P<0.05$, vs DMSO).

receptor pathway enables cells to acquire EMT, thus contributing to chemotherapy resistance to cisplatinum and taxol in ovarian cancer cell lines (15).

In our study, CTCs in human gastric cancer patients showed remarkable EMT process compared with human gastric cancer cell lines SGC-7901 and MKN-45. In the process of dissemination to peripheral blood, cells escaped the primary tumor and acquired mesenchymal composition. N-cadherin, vimentin promoted cell migration and invasion. The process of EMT occur in many tumors, such as in breast cancer (16), cervical cancer (17,18), colorectal cancer (19). EMT was regulated by many pathways involving tumor invasion and metastasis. A study showed that circulating breast tumor cells occurred in different stages of breast cancer patients, and found that patients exhibited dynamic EMT changes in CTCs (11).Others found that breast CTCs co-expressed stem cell-related markers and EMT associated markers (20,21).

Interestingly, our results found that miR-200s were downregulated by activated phosphor-Akt, and the signaling pathway was involved in EMT, migration and invasion of human gastric CTCs. The following signaling pathways involved in EMT process have been reported: notch3-zeb-EMT mediated differentiation of esophageal cancer cells (22), activation of Akt-GSK3β-Snail-EMT pathway was involved in the phenomenon of gefitinib resistance in lung cancer (23). TGF-β-p38 MAPK-EMT (24). Akt-HSF-1-Slug participated in EMT in HER2-positive breast cancer (25). AKT-snail-EMT was involved in migration of esophageal squamous cell carcinoma (26); β-catenin/tcf-zeb1 influenced EMT thereby affecting tumor cell invasion (27). LPS-NF-KB-snail-EMT (28); miR-200a-Wnt/β-catenin-zeb-EMT (29,30); activation of src induced EMT (31). TNFα-AKT/GSK-3β-Snail mediated EMT in colon cancer cells (32). Wnt3-Wnt/β-catenin signaling pathway induced EMT-like phenotype and in turn affected the trastuzumab resistance in HER2-positive breast cancer cells (33). mTOR complex 1 affects epithelial type through the opposite regulation of ZEB1/ZEB2 and miR-200b and miR-200c (34). Our study found that, p-Akt-miR200s-zeb1/twist1 mediated EMT thus affected migration and invasion of human gastric circulating tumor cells. Whether mTOR was involved in the EMT process was not assessed. In conclusion, combined with previous studies, multiple signaling pathways were involved in EMT or EMT-related transcription factors by affecting snail/slug/twist/zeb1/2, thereby affecting the biological behavior in different cells, including cell differentiation, migration and invasion and metastasis, and drug resistance.

Our study had several limitations. Four cases of CTCs were detected, more cases should be involved in the study. Another limitation is that the downstream molecular of p-Akt should be detected in order to investigate which pathway was involved in downregulation of miR-200s.

Acknowledgements

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