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Effects on Proliferation and Migration of the Human Colon Carcinoma Cell Line SW620 by Silencing of Hepatocyte Growth Factor Expression

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OBJECTIVE Hepatocyte growth factor (HGF) expression is closely related to the progression and poor prognosis of colorectal cancer patients. In this study, we investigated the effects on proliferation and migration of the human colon carcinoma cell line SW620 by silencing HGF expression.
METHODS HGF was silenced using specific HGF α/β siRNA. The proliferation, migration, cell cycle and ultrastructure of SW620 cells were examined.
RESULTS The transfection efficiency was 70%–80%. The expression rate of HGF in the experimental group was significantly lower than that in the negative and blank control groups ($P < 0.05$). The proliferation inhibition rate in the experimental group at 24, 48, 72 and 96 h after transfection was 14.2%, 50.2%, 39.5% and 23.2%, respectively. The migratory ability of cells in the experimental group was significantly inhibited compared with that in the negative control or blank control groups (58.2% *vs.* 2.1% or 0%, $P < 0.05$).
CONCLUSION The application of RNA interference to silence the expression of HGF in the colon carcinoma cell line SW620 effectively inhibits the proliferation and migration of tumor cells.

KEY WORDS: RNA interference, HGF protein, human, cellular proliferation, cell movement, colorectal neoplasms.

ABBREVIATIONS

HGF, hepatocyte growth factor; CRC, colorectal cancer; MTT, methyl thiazole tetrazolium assay; TEM, transmission electron microscopy; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide. The majority of patients tend to be in the advanced stages of cancer by the time they are admitted to hospital. However, conventional therapies involving surgery, chemotherapy and radiotherapy have been proven to be relatively ineffective. The reasons for these unsuccessful therapies are mainly due to the migration of cancer cells to distant locations via the blood and lymphatic vessels. Molecular targeted therapy has been developed rapidly in recent years,

as a result of many kinds of novel drugs being tested in clinical trials that have gained significant outcomes.

Currently, 2 kinds of targeted drugs have been approved by the Food and Drug Administration (USA) for the treatment of advanced or metastatic CRC: one is the monoclonal antibody to vascular endothelial growth factor-A, Bevacizumab (under the brand name Avastin); and the other is anti-epidermal growth factor receptor (EGFR) antibody (the fully human anti-EGFR monoclonal antibody, panitumumab, and the mouse-human anti-EGFR monoclonal antibody, cetuximab)[1,2]. These drugs have resulted in effective outcomes of the treatment for advanced CRC[3,4]. However, because the growth and metastasis of tumors are regulated by multiple targets and elements, treatment with a targeted therapy has been predicted to be ineffective or even exist a drug resistance problem[5]. Additionally, these treatment drugs can increase some side effects，such as rash, diarrhea, hypertension and cardiovascular thrombosis[5,6]. Thus, it is necessary to find new targets for CRC therapy.

Hepatocyte growth factor/scatter factor (HGF/SF) is one of the most important factors that is related to the growth and metastasis of tumors. HGF/SF is a type of polypeptide growth factor with c-Met as its receptor and a kind of transmembrane receptor tyrosine kinase. The HGF/c-Met signaling pathway plays an important role in the process of embryonic development and tissue damage repair, whilst abnormal signal transduction is closely related to the invasion and metastasis of tumor cells[7]. The HGF/c-Met signaling pathway is ubiquitously distributed in human colorectal cancer tissues and is closely related to the progression and poor prognosis of tumors[8]. HGF mRNA or protein could seldom be detected in colorectal normal tissues[9]. However, it has not been confirmed whether inhibiting HGF expression can suppress the progression of CRC.

In this study, the human colon carcinoma cell line SW620 was transfected with specific HGF α/β siRNA. Western blotting and RT-PCR were used to detect the expression of HGF protein and mRNA in cells, respectively. Proliferation assays, flow cytometry, basal lamina reconstitution, transmission electron microscopy were used to determine the effects of HGF expression on the proliferation and migration of SW620 cells.

Materials and Methods

Cell culture

The SW620 cell line was stored in the Embryology Laboratory at Hebei Medical University. Cells were grown in RPMI 1640 complete medium and incubated with 5% CO_2, at 37°C. Cells were subcultured every 2 to 3 days, using trypsin digestion to detach cells from the culture surface.

Cell transfection

Lipofectamine™ 2000 (Invitrogen, US) was used to mediate transfection. HGF α/β siRNA and control siRNA (Fluorescein Conjugate)-A were purchased from Santa Cruz (US). The experiment involved 3 groups, in which the experimental group was transfected with HGF α/β siRNA; the negative control group was transfected with the fluorescent control siRNA; and the blank control group was cultured in RPMI-1640 medium without transfection.

An appropriate number of cells were seeded onto 6 or 96-well plates 24 h before transfection in RPMI-1640 medium containing 10% fetal bovine serum. Cells were rinsed twice approximately 24 h after seeding with Opti-MEM I medium. The appropriate amount of Opti-MEM I was added to each well for further culture. The cells in the control and HGF α/β siRNAs groups were diluted in Opti-MEM I and incubated with Lipofectamine™ 2000, already diluted in Opti-MEM I, at room temperature for 20 min. The siRNA: lipid complex was added to the culture wells, mixed, and incubated with 5% CO_2, at 37°C for 6 h. The media was then discarded and replaced with RPMI-1640 medium to complete the incubation.

Fluorescing SW620 cells were observed under a fluorescent microscope 14 h following transfection and the transient transfection efficiency was determined. The transfection efficiency, V, was equivalent to the number of fluorescent cells/the number of cells in the same field of vision × 100%.

RT-PCR

TRIzol was used to extract total RNA from transfected cells after 48 h. Reverse transcription and PCR (RT-PCR) were carried out according to the manufacturer's instructions (Santa Cruz). Primer Premier 5.0 was used for primer design, and the primers were synthesized by Shanghai Shenggong Bioengineering Technical Service Ltd. Co. (China). The forward and reverse primers used in the amplification of HGF were 5'-GGT GAC CAA ACT CCT GCC A-3' and 5'-ACC TCT GGA TTG CTT GTG AAA C-3', respectively, resulting in a 300 bp amplicon. The forward and reverse primers for GAPDH (internal reference) were 5'-GGA AGG TGA AGG TCG GAG T-3' and 5'-CCT GGA AGA TGG TGA TGG G-3' respectively, resulting in a 450 bp amplicon. The cycling conditions for the PCR involved pre-denaturation step at 94°C for 3 min, followed by 30 cycles consisting of a denaturation step at 94°C for 45 s, annealing at 50°C for 45 s and elongation at 72°C for 1 min. A final extension at 72°C for 10 min was included, following the 30th cycle. PCR products were subjected to agarose gel electrophoresis. The siRNA sequence of HGF; sense 5'-CCC GUA AUA UCU UGU GCC AAA CTT-3'. antisense 5' -GUU UGG CAC AAG AUA UUA CGG GTT-3'. Control siRNA (Fluorescein Conjugate)-A were purchased from Santa Cruz (US).

Western blotting

Total protein lysates were extracted from transfected cells after 48 h, and subjected to SDS-PAGE and membrane transfer. The membrane was blocked in TTBS blocking solution (5% skim milk powder, 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) at room temperature for 2 h. The membrane was incubated with anti-HGF antibody (rabbit anti-human monoclonal antibody, 1 : 300, Upstate, Inc. US) at 4°C overnight. The membrane was washed and incubated with horseradish peroxidase conjugated secondary antibody diluted in TTBS (1 : 10,000) for 2 h at room temperature. The membrane was washed again with TTBS and a DAB coloration method was employed to detect specific binding of the primary antibody. HGF/β-actin was used to represent the relative expression level for HGF protein, and the differences between groups compared.

Methyl thiazole tetrazolium (MTT) assay

SW620 cells were seeded onto 96-well plates at a density of 2.0×10^3 cells/well with 6 replicates used in each group. The wells containing only medium material were used as blank control. The plates were assayed for MTT activity following incubation for 24, 48, 72 and 96 h, respectively. A 20 µL volume of MTT was added to each well, and cells were subjected to incubation for a further 4 h. The supernatant was discarded and 100 µL Dimethyl Sulphoxide was added to each well. Plates were subjected to shaking incubation for 20 min to fully dissolve the crystals. The blank well was used for zero adjustment, and the absorbance (A) for each well was determined at 490 nm on a plate reader. This experiment was repeated 3 times.

Flow cytometry

Flow cytometric analysis was conducted to detect the cell cycle of SW620 cells. The cells were prepared as a single suspension 48 h following transfection. A volume of 3 mL precooled 75% (v/v) ethanol was added and cells were fixed at 4°C for at least 24 h. Cells were centrifuged at 2 000 *rpm* for 7 min. The concentration of cells was adjusted to 1.0×10^6 cells/mL and 0.1 mL of this suspension containing 10% chicken red blood cells as an internal reference, was added to 1 mL propidium iodide (PI) for staining at 4°C for 30 min. The cells were filtered with a 500-sift bronze filter to obtain a qualified single-cell suspension, and then analyzed by flow cytometry (Epics-XL II; Beckman Coulter, US). The results were analyzed using Muticycle AV software. Fitting was performed for DNA in order to determine the percentage of cells in each phase of the cell cycle.

Basal lamina reconstitution

Cells were seeded onto cover slips at a density of 2.0×10^5 cells/well in a 6-well plate and incubated with 5% CO_2, at 37°C for 24 h. Cells were subjected to transient transfection, and then cultured for another 24 h. The coverslips were removed, and a blank square with clear border could be found at the position, in which the cover glasses were placed under the microscope. The cover glasses were marked with a pencil at the back side of the culture plates. The cells were cultured for a further 48 h. The 6-well plates were removed from the incubator and 5 fields of view were selected for each well at high power magnification (× 400). The number of cells presenting in the 'blank area' was counted. The migration inhibition rate in the blank control group was considered as 0%. The inhibition rate on migration ability = (the number of migratory cells in the blank control group – the number of migratory cells in the experimental group or in the negative control group)/the number of migratory cells in the blank control group × 100%.

Transmission electron microscopy

Cells were cultured in 6-well plates and transient transfection was carried out followed by subculture. Cells were dissociated into a single cell suspension, and the supernatant was discarded and 1 mL of 2.5% glutaraldehyde was slowly added to fix the cells. After that, cells were fixed with 1% osmium tetroxide for 2 h, and then rinsed, followed by graded alcohol dehydration. The samples were permeated for 1 h, at 37°C and then treated with a mixture of embedding material, acetone (3:1), at 37°C overnight; the samples were embedded, and then kept at 60°C for 48 h for polymerization. Finally the samples were subjected to ultrathin sectioning with a Leica UCT ultramicrotome and viewed with a Hitachi H-7500 transmission electron microscope.

Statistical analysis

Data were expressed as the mean ± SD. One-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between groups using SPSS 15.0 software. $P < 0.05$ was considered significant.

Results

Efficiency of HGF gene silencing on the SW620 cell line

Fluorescent control siRNA was used to transfect SW620 cells by a conventional transfection method with strong green fluorescence visible in cells after 24 h (Fig.1). The transfection efficiency was calculated to be 70%-80%.

The RT-PCR results showed that the mRNA level of HGF in the experimental group was significantly lower than those in the negative and the blank control groups ($P < 0.05$), whilst no difference was observed between the negative and the blank control groups ($P > 0.05$). GAPDH was used as an internal reference, and HGF/GAPDH represented the relative expression level of HGF mRNA. The relative expression level of HGF mRNA in the experimental group decreased by 73.0% compared with that in the blank control group, and

71.7% compared with that in the negative control group (Fig.2).

The Western blotting results were similar to those obtained from the RT-PCR. The relative expression level of HGF protein in the experimental group decreased by 56.4% compared with that in the blank control group, and 56.9% compared with that in the negative control group ($P < 0.05$). No difference was found in comparison of the relative expression level of HGF protein between the negative and blank control groups ($P > 0.05$) (Fig.3).

Effects of HGF silencing on the morphology of SW620 cells

It was found that cell proliferation in the experimental group was significantly attenuated in comparison of that in the negative and blank control groups 48 h after transfection, resulting in decreased total number of cells and significantly inhibited cell aggregation. In contrast, cells in the negative and the blank control groups grew well (Fig.4).

Effects of HGF silencing on proliferation of SW620 cells

The absorbance (A) in different groups was detected at 24, 48, 72 and 96 h, respectively, and the proliferation inhibition rates were also calculated. The proliferation inhibition rate = (the blank control group A – the experimental group A) / the blank control group A × 100%. The results showed that significant differences were found when comparing the proliferation inhibition rate in each of experimental, negative control and blank control groups at 4 different time intervals ($P < 0.05$), and the proliferation inhibition rate in the HGFα/β siRNA group at the 4 time intervals was 14.2%, 50.2%, 39.5% and 23.2%, respectively. It can be seen that the lowest proliferative ability of the cells in the experimental group presented at 48 h, indicating that the time for HGFα/β siRNA to exert its optimum inhibitory effect on HGF was 48 h after transfection. The growth curve is shown in Fig.5.

Cell cycle was determined using flow cytometry 48 h after transfection and the results were in accordance with those of MTT. The results showed that the cells in the G_0/G_1 phase accounted for (65 ± 1.7)% in the experimental group, with (28.5 ± 0.5)% cells in the S phase, indicating that HGFα/β siRNA arrested the cells at G_0/G_1 phase and reduced the number of the cells in the S phase when compared with those in the negative control group or in the blank control group ($P < 0.05$) (Table 1).

Effects of HGF silencing on the migration of SW620 cells

A basal lamina reconstitution experiment was used to assess the migratory ability of SW620 cells. The number of the cells entering the blank area in the experimental

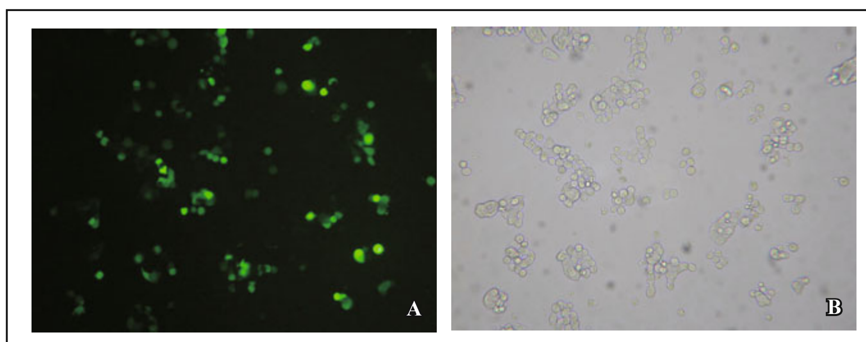

Fig.1. SW620 cells under fluorescent light (A) and ordinary white light (B) 24 h after treatment with control siRNA (× 100). Fluorescent control siRNA was used to transfect SW620 cells and relatively strong green fluorescence can be seen in 70%-80% of cells 24 h.

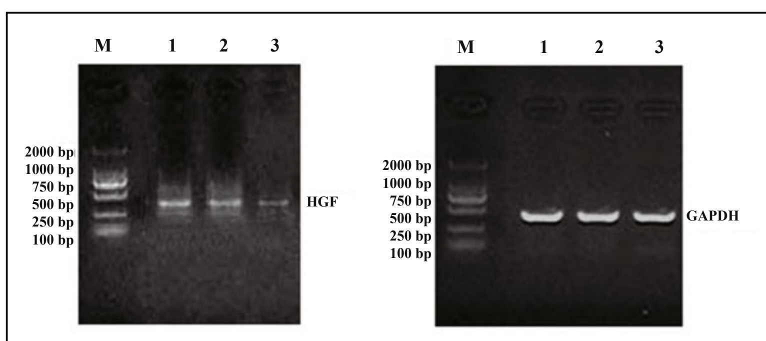

Fig.2. Relative expression level of HGF mRNA. The mRNA level of HGF in the experimental group was significantly lower than that of the negative and blank control groups ($P < 0.05$). 1, Blank control group; 2, Negative control group; 3, HGFα/β siRNA group.

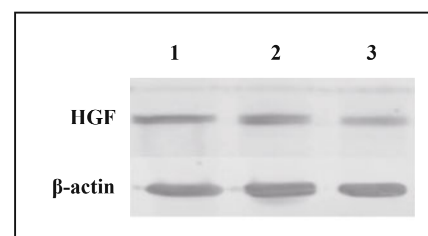

Fig.3. Relative expression of HGF protein. 1, Blank control; 2, Negative control; 3, HGF α/β siRNA.

group was significantly less than that in the negative and blank control groups, with less cell aggregation and relatively short migration distance. The migratory inhibition rate in the experimental group (60.3%) was significantly higher than that in the negative control (2.1%, $P < 0.05$) and blank control groups (0%, $P < 0.05$). No difference was found when comparing the migratory inhibition rates between the negative control group and the blank control group (Fig.6).

Table 1. Cell cycle distribution of SW620 after 48 h for transfection ($\bar{x} \pm s$, $n = 3$).

Groups	G_0/G_1 (%)	S (%)
negative	43.502 ± 1.457	39.966 ± 1.276
control siRNA	45.833 ± 1.503	37.800 ± 0.635
HGFα/β siRNA	65.033 ± 1.713▲△	28.500 ± 0.503▲△

▲$P < 0.05$ *vs*. negative group; △$P < 0.05$ *vs*. control siRNA group.

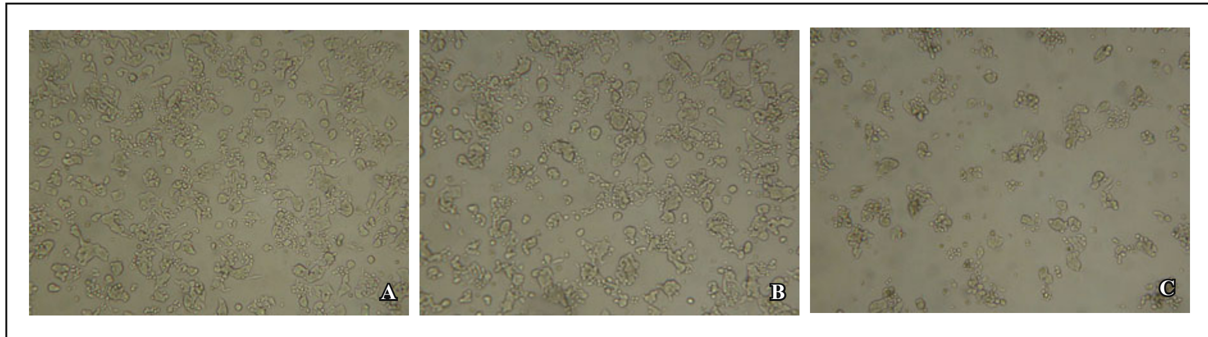

Fig.4. Effects of HGF silencing on the morphology of SW620 cells (× 100). Cell proliferation was significantly attenuated in the experimental group in comparison to the blank control group and the negative control group 48 h after transfection. A, blank control group; B, negative control group; C, HGF α/β siRNA group.

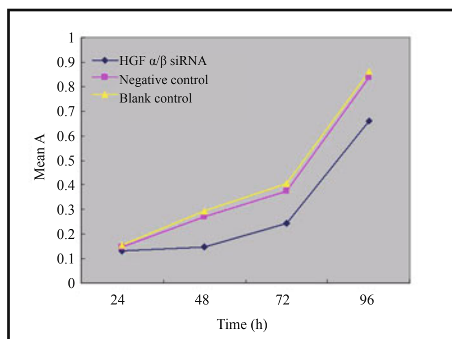

Fig.5. Growth curves of the three groups. The proliferative ability of the cells in the experimental group was weakest 48 h post-transfection. A, absorbance.

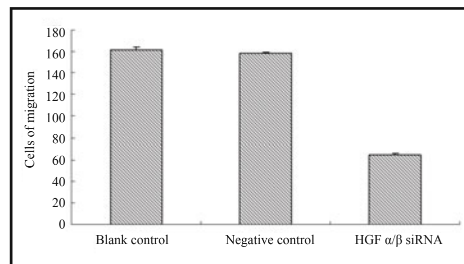

Fig.6. Effects of HGF silencing on the migration of SW620 cells (× 100). At 48 h post-transfection, the number of the cells that entered the blank area in the experimental group was significantly less than those of the negative control and blank control groups.

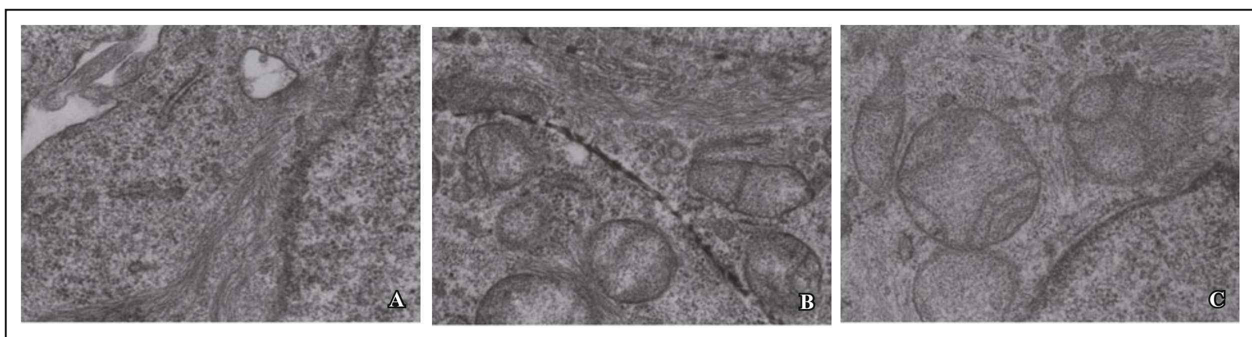

Fig.7. Effects of HGF silencing on the ultrastructure of SW620 cells as observed by TEM. A, blank control group (× 30,000); B, negative control group (× 30,000); C, HGF α/β siRNA group (× 30,000).

Effect of HGF silencing on the ultrastructure of SW620 cells

A few microfilaments were observed in the SW620 cells of the experimental group, but no microtubules were seen, whereas relatively long microtubules, in large numbers, could be found arranged in parallel lines in the paranuclear cytoplasm of cells in the negative and blank control groups. Microfilaments were found to be arranged in bundles (Fig.7).

Discussion

Colorectal cancer (CRC) is the second most common cause of cancer death in the Western world. Although both incidence and death rates for CRC have been declining over the preceding 10–20 years in Western countries, its incidence is on the rise in the Eastern areas[10,11]. At present, 5-FU-based chemotherapy is considered the standard treatment for advanced CRC. Recently, targeted agents directed at VEGF (such as bevacizumab) and EGFR (such as cetuximab and panitumumab) have become standard treatments for advanced or metastatic CRC. It has been demonstrated that combining these agents with standard treatment for CRC patients improves survival, progression-free survival and response rate compared with chemotherapy alone[3,12,13].

However, one study has shown that bevacizumab does not increase the resection rate or overall survival in patients with metastatic CRC[14]. Unfortunately, 2 recent studies have demonstrated a detrimental effect of adding cetuximab or bevacizumab to standard chemotherapy regimen[5,15]. It is well known that angiogenesis is essential for tumor growth and metastasis, but tumor nodules at early stages of malignancy only express 1 or 2 angiogenic factors, whereas advanced tumors usually produce multiple angiogenic factors. In fact, as bevacizumab has been integrated into the treatment of many different types of cancer, the development of bevacizumab-resistant tumors has become more common. Additionally, it has been shown that some factors, such as c-Met, cross react with EGFR proteins and possibly substitute for their activity, thus conferring resistance to EGFR-targeting drugs, such as cetuximab[16,17]. Therefore, it is necessary to search for novel target agents to treat CRC. HGF is a type of polypeptide growth factor, which has many functions, such as promotion of mitotic division and induction of endothelial cell migration[18]. Its receptor, c-Met, possesses tyrosine kinase activity. A disorder in the HGF/c-Met signaling pathway may induce the invasive growth of cells, and the development and metastasis of tumors. Many studies have found that the expression of HGF/c-Met is related to the invasion and metastasis of numerous types of malignant tumors, including CRC[8,19-22]. Interestingly, HGF has previously been reported to promote angiogenesis via upregulating VEGF-A[23,24]. Researchers have recently indicated that HGF also plays a critical role in lymphangiogenesis, which is associated with the lymphatic metastasis of cancer[25]. The HGF/Met pathway is potentially a new target for the treatment of CRC.

In this study, we found that silencing HGF expression significantly inhibited not only the proliferation, but also the migration of SW620 cells using specific HGFα/β siRNA technology. Previous research has shown that various tumor cells, including CRC cells, stimulate adjacent fibroblasts to secrete HGF, thereby activating c-Met via a paracrine pathway[26]. However, recent evidence has confirmed that some tumor cells, such as those from canine osteosarcoma, gastric carcinoma, hepatocellular carcinoma, and breast cancer can express HGF, and activate the c-Met receptor on the cell surface by autocrine signaling[27-30]. In the present study, we found that the human colon carcinoma cell line, SW620, can express HGF. Additionally, the proliferative and migratory ability of the SW620 cells decreases when HGF expression is knocked down, indicating that SW620 cells can secrete HGF via an autocrine mechanism and then activate the membrane receptor, c-Met.

In this study, we found that HGF α/β siRNA arrested the majority of cells transfected with HGF α/β siRNA in G_1 phase, and the number of cells in S phase decreased. This indicates that RNAi inhibits cell proliferation by inhibiting the expression of HGF, and thus, restrains the process of mitotic division in cancer cells. This result is consistent with those in the previous reports[31].

It is well known that tumor cell motility plays a crucial role in the development of tumor metastasis and is affected by a variety of factors. HGF is one of these factors and stimulates human colon carcinoma cell migration remarkably[26]. These experiments demonstrate that silencing HGF can reduce the number of actin filaments and microtubules in cells. While the mechanism of how silencing HGF expression inhibits the invasion of cancer cells is currently unknown, it is doubtless that the basis for cell migration involves the cytoskeleton, which includes actin filaments and microtubules. The elongation of actin filaments and polarized growth of microtubules play important roles in the process of cell migration. Previous studies have shown that HGF can phosphorylate Src protein after binding to c-Met. The Src protein, thus, induces the re-arrangement of the cytoskeleton, resulting in CRC tumorigenesis and liver metastasis[32].

In summary, we found that the application of RNAi to silence the expression of HGF effectively inhibits the proliferation and migration of human colon carcinoma cells. However, the exact mechanisms need to be further investigated.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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