# Alpha-fetoprotein can promote gastric cancer progression via upregulation of metastasis-associated colon cancer 1

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**Abstract.** High serum alpha-fetoprotein (AFP) level is a predictor of poor prognosis in patients with gastric cancer (GC). AFP-producing GC (AFP-GC) is an aggressive subtype of GC characterized by a high incidence of liver metastasis and high c-Met expression. High expression of metastasis-associated colon cancer 1 (MACC1), which is the transcription activator of c-Met, also predicts a poor prognosis of GC. c-Met is known to be involved in tumor progression into malignant invasive phenotypes. Considering that high c-Met expression is simultaneously positively correlated with high AFP and MACC1 expression levels and that high expression of AFP or MACC1 predicts poor prognosis in GC, we hypothesized that an interaction may exist between AFP and MACC1. In the present study, GC cell lines with AFP-overexpression, MACC1-downregulation and the combination of both transfections were used as experimental models. The relative mRNA and protein expression of c-Met, AFP and MACC1 were analyzed using reverse transcription quantitative PCR and western blotting, respectively. Cell viability was evaluated using Cell Counting Kit-8 assay. Cell invasion and cell migration were examined using Transwell migration assay with and without Matrigel, respectively. The results demonstrated that, compared with the control group, the mRNA and protein expression of MACC1was significantly elevated in the AFP-overexpressed group and in the group with AFP overexpressed and MACC1 downregulated. Furthermore, a significantly enhanced cell viability, migration and invasion were observed in the AFP-overexpressing group, whereas opposite effects were found in the MACC1-downregulating group. In summary, the results from this study indicated that AFP may promote GC progression by stimulating MACC1. This finding may help illustrating the aggressive behaviors of GC in patients with high AFP serum level and AFP-GC.

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*Abbreviations:* AFP, alpha-fetoprotein; AFP-GC, AFP-producing gastric cancer; MACC1, metastasis-associated colon cancer 1; GC, gastric cancer; HGF, hepatocyte growth factor

*Key words:* alpha-fetoprotein, metastasis-associated colon cancer, gastric cancer, cancer progression, alpha-fetoprotein-producing gastric cancer

## Introduction

Gastric cancer (GC) is currently one of the most common malignant tumors diagnosed and is the third leading cause of cancer-associated mortality worldwide (1,2). Alpha-fetoprotein-producing GC (AFP-GC) is more aggressive with a low incidence of 1.2-15% (3,4) compared with non-AFP GC. At a comparable stage, patients with AFP-GC displayed significantly lower survival rates and higher incidence of lymphatic metastasis, liver metastasis and vascular invasion than those with non-AFP GC (5,6).

Although the aggressive behaviors of AFP-GC have drawn much attention, whether and how AFP could regulate GC progression remain unknown. AFP is a glycoprotein produced to a lesser extent in the fetal gastrointestinal tract but mainly produced by the yolk sac and liver during fetal development (7). It has been reported that in patients with common GC, a high serum AFP level is considered as an independent predictor of high metastasis and poor prognosis (8,9).

Amemiya *et al* (10) reported that patients with AFP-GC have higher expression of c-Met compared with those with non-AFP-GC. c-MET protein is encoded by the MET proto-oncogene and its high-affinity ligand is the hepatocyte growth factor (HGF). Previous studies indicated that the HGF-Met signaling pathway plays a vital role in the growth, metastasis and drug resistance in gastrointestinal cancers (11,12). Metastasis-associated colon cancer-1 (MACC1) gene, which is ac-Met transcriptional regulator, has been identified as a colon cancer oncogene that could promote metastasis (13). Higher expression of MACC1 was found in GC tissues compared with adjacent non-tumor tissues (14,15) and is associated with distant metastasis and low survival rate (15-21). Furthermore, the expression of MACC1, HGF and c-Metis positively correlated with each other in GC tissues (14,15).

Considering that high c-Met expression is correlated with high AFP and MACC1 expression levels, both predict poor prognosis in GC, we hypothesized that an interaction may exist between AFP and MACC1 activity, which might subsequently enhance GC progression. The present study aimed to investigate this interaction *in vitro* models mimicking AFP-GC.

## Materials and methods

*Cell lines*. The human GC cell lines, MKN-45 (cat. no. JCRB0254) and GCIY (Cat. No. TKG0405) were purchased from Biofeng. MKN-45 was cultured in RPMI-1640 medium (HyClone; Cytiva; cat. no. SH30809.01) and GCIY was cultured in Minimum Essential Media (MEM) medium (HyClone; Cytiva; cat. no. SH30024.01), respectively, and both were supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.; cat. no. 10270-106) and 1% of 10,000 U/ml penicillin-streptomycin (Thermo Fisher Scientific, Inc.; cat. no. 15140122). All cells were placed at 37˚C in a humidified incubator containing 5% $CO_2$. Kataoka *et al* (22) reported the abundant expression of AFP in GCIY cells and the low expression of AFP in MKN-45 cells. Therefore these two cell lines were used in the study.

*Cell transfection*. To overexpress AFP, AFP human untagged clone (OriGene; cat. no. SC122582) was used and combined with pCMV6-XL5 vector (OriGene Technologies, Inc.). For downregulation of MACC1, short hairpin (sh)RNA targeting MACC1 (shMACC1; GeneCopoeia, Inc.; cat. no. HSH009476) was integrated into the pSUPER-retro-puromycin plasmid (GeneCopoeia, Inc.). The sequence of shMACC1 was 5'-AAG AUUGGACUUGUACACUGC-3' and the sequence of the negative control (NC) shRNA was 5'-UUCUCCGAACGUGUCACG UTT-3'. Transfections were performed using Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Inc.; cat. no. L3000008) in 37˚C for 48 h unless otherwise specified. Subsequent experiments were performed 48 h after transfection.

*Reverse transcription quantitative (RT-q) PCR*. Total RNA was extracted from cells using TRIzol® Reagent (Thermo Fisher Scientific, Inc.; cat. no. 15596-026) according to the manufacturer's instructions. Briefly, the sample was homogenized with TRIzol® and then chloroform (Sinopharm Chemical Reagent Co., Ltd.; cat. no. 10006818) was added. The homogenate was left to stand for at least 5 min at room temperature to allow its separation into an RNA-containing aqueous phase and a lower organic layer. RNA was then precipitated from the aqueous layer by adding isopropanol (Sinopharm Chemical Reagent Co., Ltd.; cat. no. 80109218). Extracted RNA was dissolved in RNase-free-d$H_2O$. The quantity and quality of extracted RNA were measured and confirmed using NanoDrop 2000 (Thermo Fisher Scientific, Inc.), and 1 $\mu$g of total RNA was reverse transcribed into cDNA using commercial PrimeScript™ RT Master Mix (Perfect Real Time; Takara Bio, Inc.; cat. no. RR036A) according to the manufacturer's instructions. Briefly, RNA was diluted to adequate concentrations and added to 2 $\mu$l 5X PrimeScript RT Master Mix (Perfect Real Time) and RNase-free-d$H_2O$ was added up to 10 $\mu$l. The reaction was achieved using Veriti™ 96-Well Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.; cat. no. 4375786) according to the following conditions: Incubation at 37˚C for 1 h, then termination at 85˚C for 5 min to inactivate the enzymes. After the termination reaction, the product was kept at 4˚C, after which qPCR was performed using TB Green® Premix Ex Taq™ II (TliRNaseH Plus; Takara Bio, Inc.; cat. no. RR820A). The reaction was completed using Applied Biosystems 7300 Real-Time PCR System (Thermo Fisher Scientific, Inc.) and the conditions of the reaction were as follows: 95˚C for 30 sec, 95˚C for 5 sec, and 60˚C for 34 sec (1 cycle), for a total of 40 cycles. β-actin was used as the endogenous control. The relative expression levels were normalized to endogenous control and were expressed as $2^{-\Delta\Delta Cq}$ (23). The sequences of the primers are listed in Table SI.

*Western blotting*. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B) containing 1% phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology; cat. no. ST506) and 1X Protease inhibitor cocktail (Beyotime Institute of Biotechnology; cat. no. P1005) on ice. Protein concentration was quantified by Pierce BCA protein assay. Proteins (20 $\mu$g) were separated by 10-15% SDS-PAGE and were transferred onto PVDF membranes (Sangon Biotech, Co., Ltd.). Then the membranes were washed with dd$H_2O$ for 2 min with shaking, rinsed with dd$H_2O$ twice, and incubated in SuperSignal™ Western Blot Enhancer (Thermo Fisher Scientific, Inc.; cat. no. 46640) for 10 min with shaking at room temperature to enhance detection according to the manufacturer's instructions. The membranes were blocked in 1% milk in 0.05% Tween-20 in TBST buffer at room temperature for 1 h after washed with dd$H_2O$ for 5 times. Then membranes were incubated with primary antibody diluted in the Primary Antibody Diluent from the enhancer kit (1:1,000) against c-Met monoclonal antibody (Invitrogen; cat. no. 37-0100), MACC1 monoclonal antibody (Abcam; cat. no. ab242199), alpha-fetoprotein monoclonal antibody (Abcam; cat. no. ab3980) and β-actin monoclonal antibody (Thermo Fisher Scientific, Inc.; cat. no. AM4302) for 1 h at room temperature. After washing three times for 5 min with TBST buffer, membranes were incubated with HRP-labeled Goat-Anti-Mouse IgG secondary antibody (H+L; Beyotime Institute of Biotechnology; cat. no. A0216) at room temperature for 30 min. After washing membranes four times for 5 min, enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.; cat. no. 32106) was applied to detect the signal on the membranes. The data were analyzed via densitometry using Gel-Pro Analyzer Gelpro 32 software (Analytik Jena AG) and normalized to expression of the internal control β-actin.

*Cell Counting Kit-8 (CCK-8) cell proliferation assay*. Cell proliferation was determined using the CCK-8 assay purchased from Dojindo Molecular Technologies, Inc. (cat. no. CK04). Cells were seeded at a density of $5x10^3$ cells per well in a 96-well plate for 24 h and were transfected with overexpression vector and/or shRNA, and further incubated for 24, 48 and 72 h. Subsequently, at 1 h before the endpoint of incubation, 10 $\mu$l CCK-8 reagent was added to each well for 1 h. Absorbance was read at 450 nm using a microplate reader.

*Cell invasion and migration assay*. The cell invasion and migration were assessed using 6.5 mm Transwell® migration assay, with 8.0 $\mu$m Pore Polycarbonate Membrane Insert, Sterile (Corning, Inc.; cat. no. 3422). For cell invasion, serum-free medium was mixed with the BD Matrigel™ hESC-qualified Matrix (BD Biosciences; cat. no. 354277) in a 1:10 ratio. This mixture (50 $\mu$l) was added to the bottom of the insert. The Matrigel was then incubated at 37˚C for 4 h to solidify. Then, $5x10^4$ cells were transfected and at 24 h following transfection,

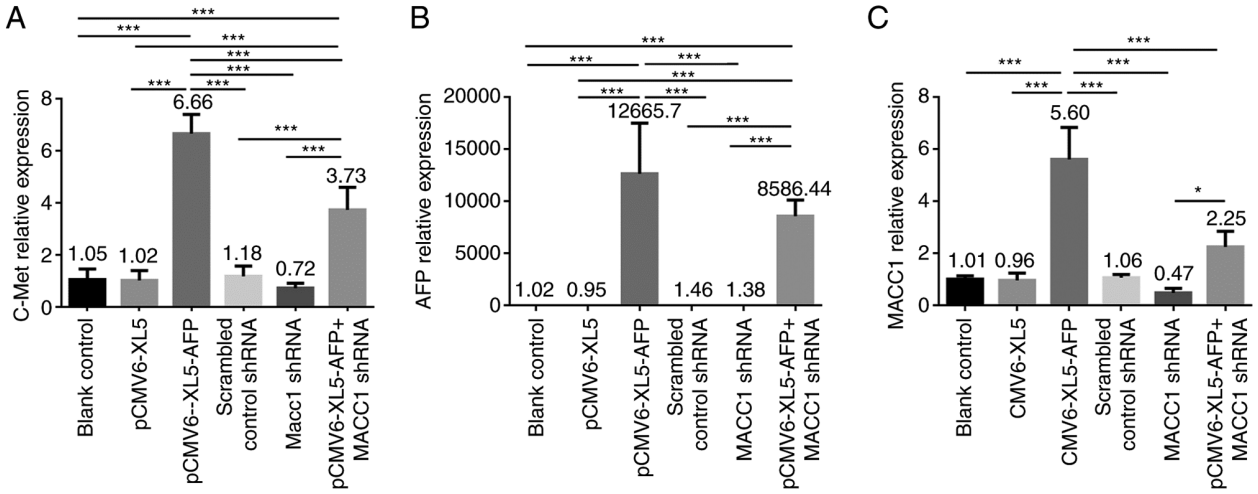


Figure 1. Relative mRNA expression level of (A) c-Met, (B) AFP and (C) MACC1 in the MKN-45 cell line. *P<0.05 and ***P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

cells were harvested by trypsinization, washed with serum-free medium and placed in the upper chamber of the Transwell. The lower chamber contained 500 $\mu$l medium supplemented with 10% FBS that was used as chemo-attractant. After incubation at 37˚C with 5% $CO_2$ for 48 h, the cells in the inner side of the chamber were removed using cotton swabs. Invaded cells on the lower membrane surface were fixed with methanol for 15 min at room temperature and stained with 0.1% crystal violet for 10 min at room temperature. Images of the invaded cells were taken using a light microscope (Olympus IX71, x200 magnification) and cell numbers were counted. Cell migration assay was performed in a similar way except that $1x10^5$ cells were added into the insert without Matrigel pre-coating. Each experiment was conducted in triplicate and repeated three times.

*Statistical analysis*. Data analysis was performed using SAS version 9.4 software (SAS Institute, Inc.). Comparison among multiple groups was performed using one-way ANOVA followed by Tukey's post-hoc test. Data in Fig. 5 were analyzed by two-way ANOVA followed by Tukey's post-hoc test, with group and post-transfection time considered as independent variables. All data were presented as the means ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

## Results

*MACC-1 is upregulated following AFP overexpression*. We used 10% FBS in the medium as indicated in the culture protocol of the cell line. The additional effect of 10% FBS on growth can be ruled out by comparing experimental groups with the respective controls.

To investigate whether the mRNA level of c-Met, AFP and MACC1 are regulated following AFP overexpression or MACC1 downregulation, RT-qPCR was performed on MKN-45 cell untreated or transfected with empty vector (pCMV6-XL5), pCMV6-XL5-AFP, scrambled control shRNA, MACC1 shRNA and pCMV6-XL5-AFP+MACC1 shRNA. The mRNA expression level of c-Met was significantly elevated in AFP-overexpressed and AFP-overexpressed + MACC1-downregulated groups compared with the control group (Fig. 1A; both P<0.001), and was significantly higher in AFP-overexpressed group compared with AFP-overex pressed + MACC1-downregulated group (Fig. 1A; P<0.001).No significant difference in mRNA expression level of c-Met was observed in MACC1-downregulated group (Fig. 1A,). The higher expression level of AFP was observed in the AFP-overexpressed and AFP-overexpressed + MACC1-downregulatedgroups as seen in Fig. 1B (both P<0.001). An elevated mRNA level of MACC1 was detected in both AFP-overexpressed and AFP-overexpressed + MACC1-downregulated groups (Fig. 1C). Furthermore, the mRNA level of MACC1 was significantly increased in AFP-overexpressed + MACC1-downregulated group compared with MACC1-downregulated group (Fig. 1C; P<0.05).

To further investigate the expression of the above proteins after AFP overexpression or MACC1 downregulation, western blotting was performed with the same approaches as in the RT-qPCR experiments (Fig. 2A). In MKN-45 cells, the protein expression of c-Met was significantly higher in AFP-overexpressed group than MACC1-downregulated group (Fig. 2B; P<0.05). The relative protein expression of AFP was higher in AFP-overexpressed and AFP-overexpressed + MACC1-downregulated groups (Fig. 2A and C; P<0.001), while that of MACC1 was significantly elevated in the AFP-overexpressed group (Fig. 2A and D; P<0.001). Furthermore, protein expression of MACC1 was significantly higher in AFP-overexpressed + MACC1-downregulated group compared with MACC1-downregulated group (Fig. 2A and D; P<0.001), suggesting MACC1 was upregulated by AFP.

We also investigated the expression levels of c-Met, AFP, and MACC1 in the GCIY cell line using the same approaches. The mRNA level of c-Met compared with controls was significantly elevated in AFP-overexpressed group (Fig. 3A; P<0.05), and no significant difference was displayed in MACC1-downregulated group (Fig. 3A). The higher expression level of AFP was observed in AFP-overexpressed and AFP-overexpressed + MACC1-downregulated groups (Fig. 3B; both P<0.001), and a significantly elevated mRNA level of MACC1 was detected in both AFP-overexpressed and AFP-overexpressed + MACC1-downregulated groups (Fig. 3C; P<0.001). In the GCIY cell line, the expression

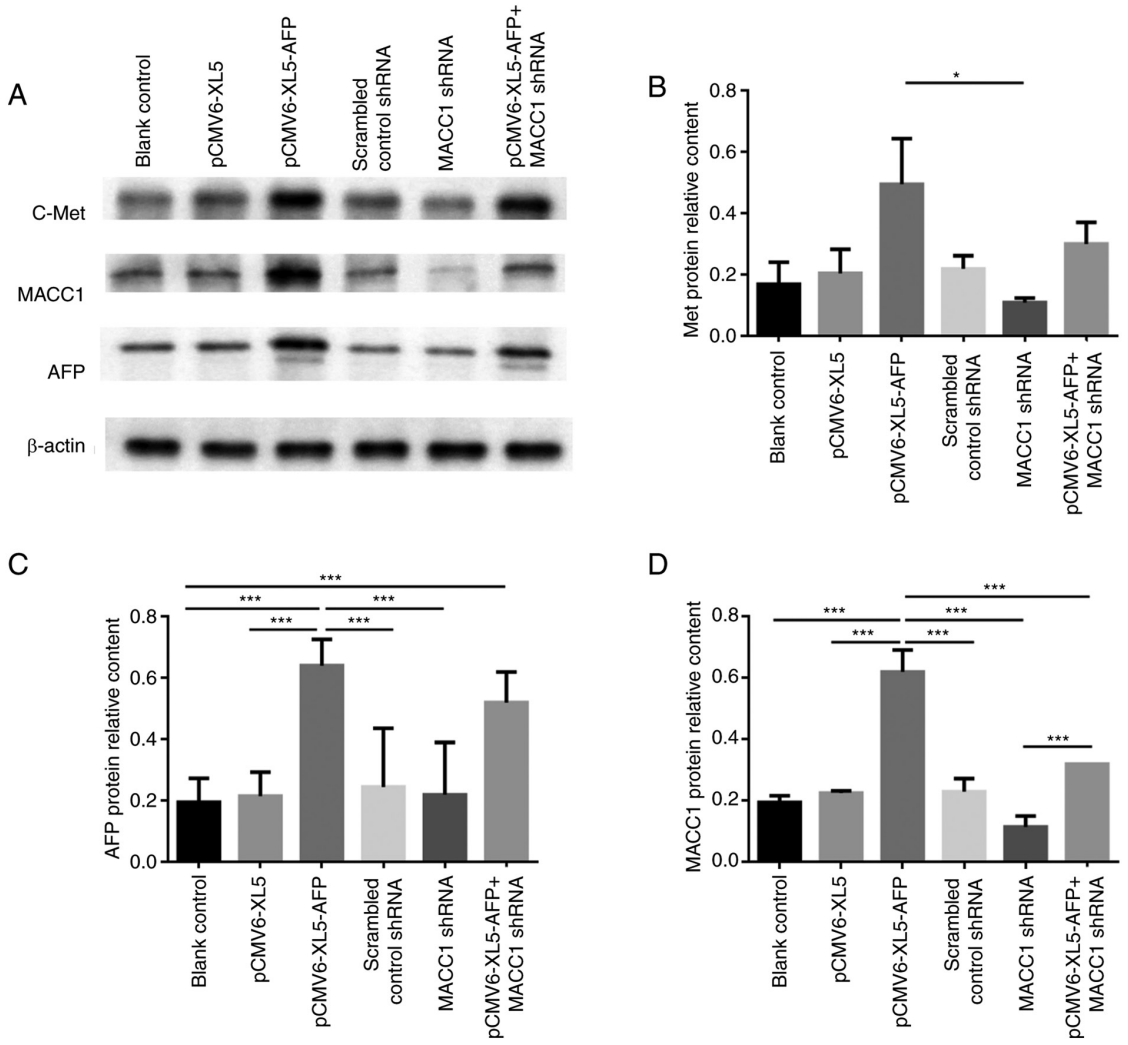


Figure 2. Protein expression of c-Met, AFP and MACC1 in the MKN-45 cell line. (A) Representative western blotting data. Relative expression of (B) c-Met, (C) AFP and (D) MACC1. *P<0.05 and ***P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

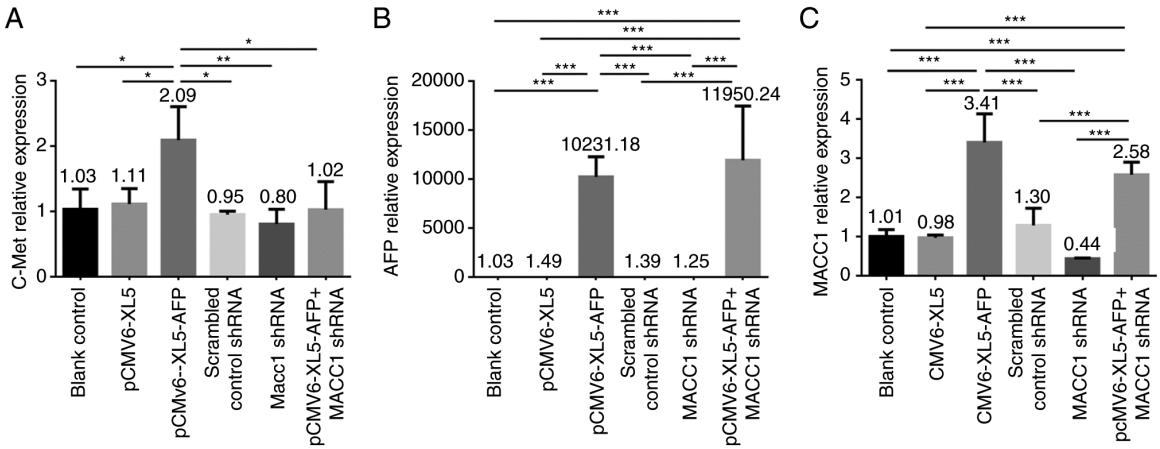


Figure 3. Relative mRNA expression level of (A) c-Met, (B) AFP, and (C) MACC1 in the GCIY cell line. *P<0.05, **P<0.01 and ***P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

level of c-Met compared with control group was significantly increased in the AFP-overexpressed group (Fig. 4A and B; P<0.001). A significantly higher protein expression level of AFP was detected in both AFP-overexpressed and AFP-ove rexpressed + MACC1-downregulated groups (Fig. 4A and C; P<0.001), and that of MACC1 was significantly elevated in the AFP-overexpressed group (Fig. 4A and D; P<0.001). In addition, the protein expression of MACC1 was significantly

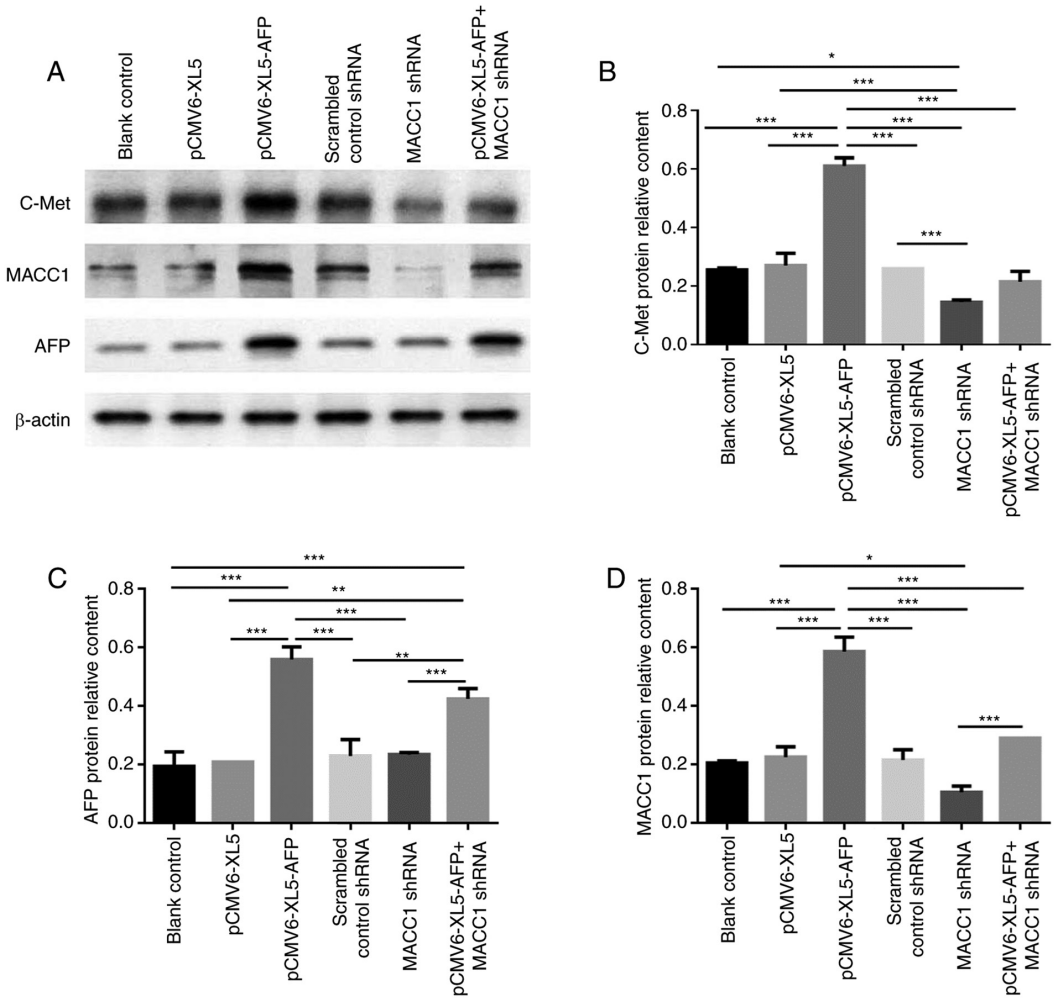


Figure 4. Protein expression of c-Met, AFP and MACC1 in the GCIY cell line. (A) Representative western blotting data. Relative expression of (B) c-Met, (C) AFP and (D) MACC1. *P<0.05, **P<0.01 and ***P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

higher in AFP-overexpressed + MACC1-downregulated group compared with MACC1-downregulated group (Fig. 4A and D; P<0.001), suggesting that AFP could upregulate MACC1.

*Regulatory effect of AFP overexpression or MACC1 downregulation on GC cell progression*. CCK-8 assay was performed in MKN-45 cells to analyze the effects of AFP overexpression or MACC1 downregulation on cell proliferation. Cells were treated and grouped similarly as in the above experiments, except that the scrambled control shRNA group was omitted. Fig. 5 presents the relative cell viability normalized to control group at 24, 48 and 72 h following transfection. The results demonstrated that both transfection time and group were significant sources of variation (both P<0.001), and post-hoc Tukey's test comparison indicated that there were differences between groups (P<0.001). Compared with blank control group at 48 and 72 h, cell viability was significantly increased in the AFP-overexpressed group (P<0.001). A significantly decreased cell viability was observed in the MACC1-downregulated group at all three time points (P<0.001). Compared with AFP-overexpressed + MACC1-downregulated group, cell viability was significantly lower in MACC1-downregulated group (P<0.001) but higher in AFP-overexpressed group (P<0.001) at the three time points.

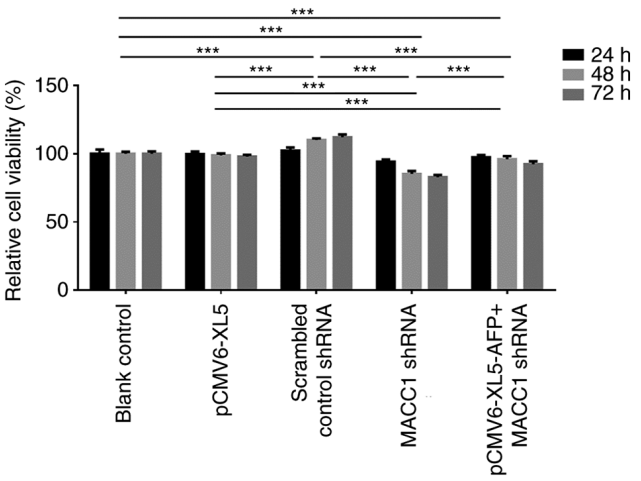


Figure 5. Cell viability assessed with the Cell Counting Kit-8 assay at 24,48 and 72 h following the indicated transfections. Relative cell viability was expressed as a percentage compared with the blank control defined as 100%. ***P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

These results suggested that AFP and MACC1 may enhance cell viability.

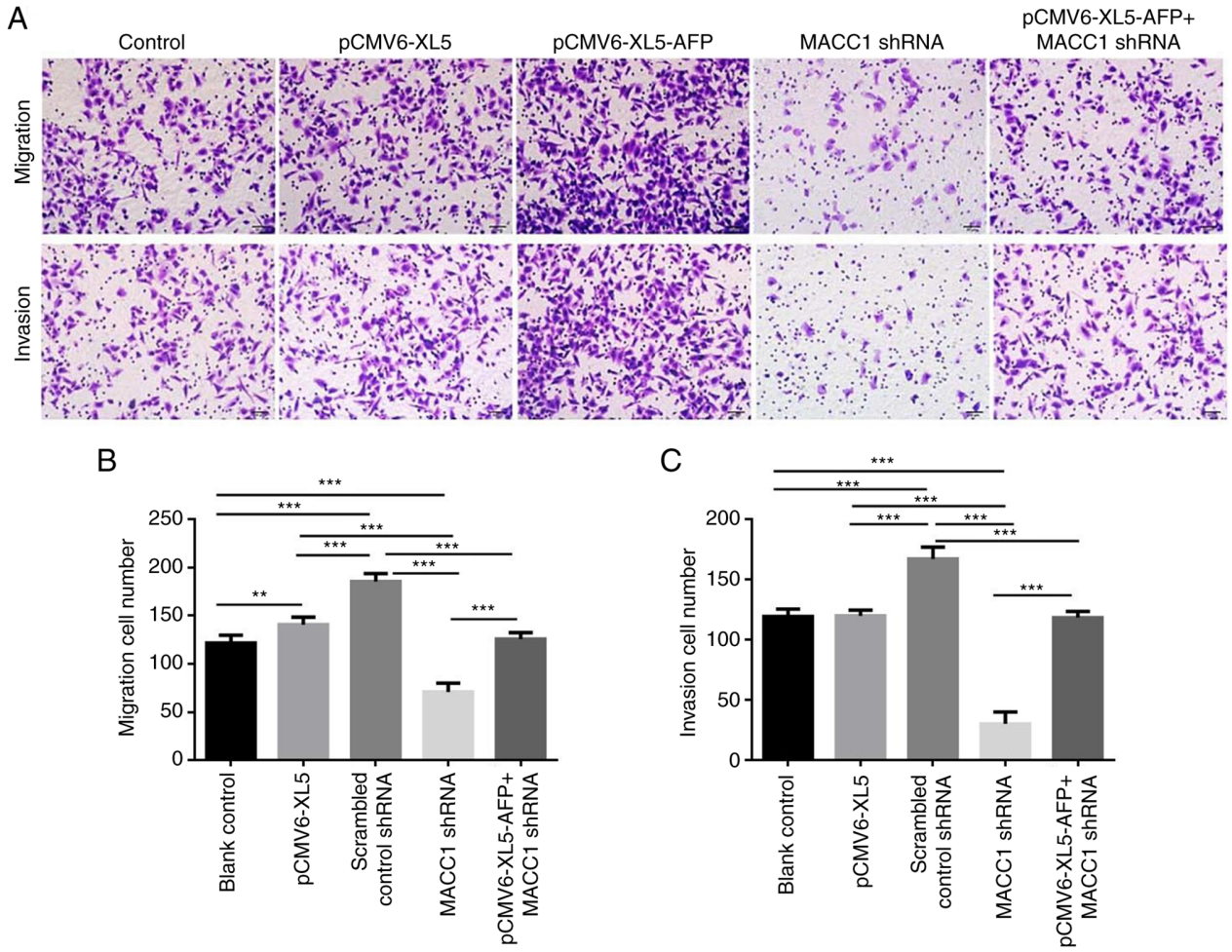


Figure 6. Cell migration and invasion analysis following various transfections in MKN-45 cells. (A) In cell migration and invasion, cells were stained with 0.1% crystal violet, photographed and the cell number was counted (200x magnification). (B) Number of migrating cells in each treatment group. (C) Number of invasive cells in each treatment group. Scale bar, 50 $\mu$m. $^{**}$P<0.01 and $^{***}$P<0.001. AFP, alpha-fetoprotein; MACC1, metastasis-associated colon cancer 1; sh, short hairpin.

The effect of AFP overexpression or MACC1 downregulation on cell migration and invasion was also evaluated as presented in Fig. 6A. Compared with the control group, the number of migrated and invaded cells was significantly increased in the AFP-overexpressed group and AFP-overexpressed + MACC1-downregulated group (Fig. 6B and C; P<0.001).

## Discussion

To the best of our knowledge, the present study was the first to reveal that AFP could upregulate MACC1 activity and thus promote GC proliferation, migration, and invasion. Numerous previous studies reported that high expression levels of AFP and MACC1 are significantly associated with higher metastasis and poor prognosis in GC, respectively (8,9,14,15,24,25). The findings from the present study demonstrated that AFP could enhance tumor progression rather than acting as a tumor marker. This may help illustrating the aggressive behaviors of AFP-GC and common GC in patients with high AFP serum levels.

Serum AFP level is a prognostic factor for overall survival and treatment response in patients with AFP-GC (24,25). In common GC, a higher serum AFP level is also an independent factor of poor prognosis (8,9). However, the underlying molecular mechanism of AFP in GC progression remains unclear. A previous study reported that activation of the Wnt signaling pathway is responsible for stimulation of cell proliferation and aggressiveness enhanced by AFP in AFP-overexpressed GC cells (26). The findings from the present study demonstrated the enhancement of GC progression by AFP via upregulation of MACC1. To further confirmation this results, the correlation between AFP and MACC1 expression levels should be evaluated in human GC tissues. Additional research is therefore needed to determine the molecular mechanism underlying AFP-MACC1 regulation on GC progression.

High expression levels of MACC1 in GC is significantly associated with poor prognosis (15-21). However, it remains unknown why the malignant progression is accompanied by upregulation of MACC1. It has been reported that GC cells escape from glucose deprivation by lowering the expression of RhoA-specific GTPase-activating protein DLC3 to upregulate its downstream target MACC1 (27), suggesting that MACC1 upregulation may respond to metabolic stress. Considering that the number of proliferated cells as well as MACC1 expression level were significantly higher in the AFP-overexpressed group compared with the control in the

present study, we hypothesized that MACC1 upregulation may result from metabolic stress because of an energy deficit due to uncontrolled cell proliferation enhanced by AFP.

The results from the present study performed on two GC cell lines showed that AFP upregulated MACC1 activity, regardless of cellular c-Met protein expression levels. The inconsistency between the mRNA and protein expression of c-Met and AFP may be associated with a lack of certain post-transcriptional factors in *in vitro* cell line systems. However, further investigation is required to clarify the underlying mechanisms. According to the results from the present study, AFP overexpression enhanced GC cell viability, migration and invasion, thus contributing to GC progression. Furthermore, knockout of MACC1 by shRNA attenuated GC cell viability, migration and invasion. GC progression may therefore be enhanced following activation of MACC1.

HGF-Met signaling pathway plays an important role in the proliferation and metastasis of gastrointestinal cancers (11,12). High c-Met expression has been reported in AFP-GC tissues (10) but not in non-AFP GC tissues. The positive rate of c-Met in common GCs ranges from 18 to 71.1% (26-30). The gene amplification of c-Met is positively correlated with the cancer stage, and c-Met was found to be overexpressed in GCs with deeper invasion and distant metastasis (30,31). A higher c-Met expression level has been observed in GC with liver metastasis compared with the other primary tumor types (30). In addition, a positive correlation between MACC1 and c-Met expression was reported in GC tissues (15,16), and MACC1 was demonstrated to mediate c-Met phosphorylation in the HGF/c-Met signaling pathway in GC (32). However, further investigation is required to clarify whether and how c-Met might be involved in AFP-MACC1 regulation on GC progression, especially to determine the following features: i) Phosphorylated c-Met level in AFP-overexpressed and control GC cells; and ii) expression level of AFP and MACC1 when nerve growth factor is upregulated in GC cells.

This study presented some limitations. Firstly, co-expression of AFP and MACC1 should be evaluated in human GC samples to identify the existence of an AFP-MACC1 pathway. Secondly, the level of phosphorylated c-Met in AFP-overexpressed and control GC cells should be evaluated to determine whether c-Met might be involved in AFP-MACC1 pathway. Thirdly, a colony formation assay should be performed to validate the data from CCK8 assay.

In summary, the present study demonstrated that both AFP and MACC1 enhance cell viability, migration and invasion of GC cells. In addition, AFP may enhance GC progression via upregulating MACC1 activity. Considering that AFP and MACC1 are predictors of poor prognosis in patients with GC, further investigating this AFP-MACC1 regulation may provide theoretical information for the development of therapeutic approaches for GC.

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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**

FL designed the study, reviewed the manuscript and guaranteed the integrity of the entire study. XM performed literature research, experimental studies and data acquisition and prepared the manuscript. JW analyzed data, performed statistical analysis and edited the manuscript. FL and XM confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interest.

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