

Inhibition of apoptosis by *miR-122-5p* in α -fetoprotein-producing gastric cancer

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Abstract. α -Fetoprotein (AFP)-producing gastric cancer (AFPGC) is recognized as one of the most aggressive tumors with subsequent poor prognosis compared with common gastric cancer (GC) subtypes. However, the molecular mechanism remains to be elucidated. We previously identified that *miR-122-5p* could be a useful biomarker in AFPGC patients. We examined herein the biological function of *miR-122-5p* and the molecular mechanism underlying tumor progression in AFPGC. We used the AFPGC cell line (FU97) and *miR-122-5p* inhibitor to examine the function of *miR-122-5p*. Moreover, we investigated the possible targets of *miR-122-5p*. The expression level of *miR-122-5p* was significantly increased in the FU97 cell line than in common GC cell lines. Also, suppression of *miR-122-5p* significantly reduced AFP levels and proliferation in AFPGC through an induction of apoptosis. Western blotting revealed that the expression of anti-apoptotic protein (Bcl-2) was decreased and that of pro-apoptotic protein (caspase-3) was increased in *miR-122-5p* suppression of FU97. Moreover, we revealed that FOXO3 was an important target of *miR-122-5p* in AFPGC, which inhibited apoptosis and subsequently manifested aggressiveness. In conclusion, *miR-122-5p* inhibited apoptosis and facilitated tumor progression by targeting FOXO3 in AFPGC, which indicates the possibility of *miR-122-5p* as a potential therapeutic target in AFPGC.

Introduction

Gastric cancer (GC) is one of the most common solid tumors and the third leading cause of cancer-related deaths

worldwide (1,2). Despite improvements in treatment approaches, prognosis of patients with advanced GC remains poor even after curative resection.

Among various tumor subtypes, α -fetoprotein (AFP)-producing GC (AFPGC) is recognized as a most aggressive tumor with subsequent poor prognosis compared with common GC subtypes, with a high propensity for liver metastasis (3-7). AFPGC has been reported to have high proliferative, proangiogenic and reduced apoptotic activity by immunohistochemical examination (7). However, the molecular mechanism remains to be elucidated.

Among various molecules, microRNAs (miRNAs) have attracted attention as having oncogenic or tumor-suppressive functions and also tumor progression-related factors. miRNAs are endogenous, small, non-coding, single-stranded RNAs of 20-25 nucleotides that regulate the expression of target genes at the post-transcriptional level by binding to complementary sequences (8). A single miRNA can influence the expression of hundreds of genes. Therefore, we hypothesized that some specific miRNAs may play crucial roles in AFPGC. Previously, we demonstrated that *miR-122-5p* was significantly higher in AFPGC tissues and that the plasma expression level may be a useful biomarker in patients with AFPGC (9). In the present study, we examined the biological function of *miR-122-5p* and the molecular mechanism underlying tumor progression in AFPGC.

Materials and methods

Culture of GC cell lines and primary tumor samples. The AFPGC cell line FU97 and the other GC cell lines MKN7, MKN74 and NUGC-3 were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). FU97 was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.), penicillin-streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 10 mg/l insulin (Sigma-Aldrich; Merck KGaA). The other GC cell lines were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and penicillin-streptomycin

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(Sigma-Aldrich; Merck KGaA). All cells were cultured in 5% carbon dioxide at 37°C in a humidified chamber.

All primary AFPGC and non-AFPGC samples at various stages as controls from the same cohort were obtained from 25 patients (18 males and 7 females) who underwent gastrectomy for GC at the University of Yamanashi Hospital between January 2012 and December 2018 (age, 68.04±10.97 years). Tumor specimens and resected lymph nodes obtained at surgery were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin after fixation. AFPGC was defined on the basis of a plasma AFP level >10 ng/ml or positive AFP immunoreactivity in tissue samples.

The present study was approved by the Ethics Committee of the Yamanashi University and was performed in accordance with the ethical standards of the Declaration of Helsinki and its amendments. Informed written consent was obtained from all patients.

Transfection of FU97 with miRNA inhibitors. The miRNA inhibitors for *miR-122-5p* (cat. no. MH11012) (UGGAGUGUG ACA AUGGUGUUUG) and negative control anti-miRNA (mirVana miRNA Inhibitor Negative Control #1) (UUACGU CGUCGCGUCGUUAU) were purchased from Thermo Fisher Scientific, Inc. miRNA inhibitors were transfected into cells at the indicated concentration using Lipofectamine RNAiMAX (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfection concentration of *miR-122-5p* inhibitor and negative control used in the present study was 30 nM. Total RNA and protein were subsequently collected at 48 h following transfection.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was extracted using the miRNeasy Mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols. Subsequently, a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.) was used to measure the total RNA concentration. The reverse transcription reaction was conducted with a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Total RNA and miRNA levels were quantified by qRT-PCR according to standard procedures. qRT-PCR conditions were as follows: Pre-heating for 10 min at 95°C; repeating 40 cycles at 95°C for 15 sec and 60 sec at 60°C. Total RNA levels were normalized to the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and miRNA levels were normalized to *RNU6B*. The following primers were used for the TaqMan assay (Thermo Fisher Scientific, Inc.): *AFP* (cat. no. Hs01040598_m1), forkhead box O3 (*FOXO3*; cat. no. Hs00818121_m1), human *hsa-miR-122-5p* (cat. no. 002245) (UGGAGUGUGACA AUGGUGUUUG), *GAPDH* (cat. no. Hs02786624_g1) (forward, 5'-AATGGACAA CTGGTCGTGGAC-3' and reverse, 5'-CCCTCCAGGGGA TCTCTGTTT-3') and *RNU6B* (cat. no. 001093). qRT-PCR was performed in triplicate. Δ Cq values for all RNAs relative to the control gene *GAPDH* and *RNU6B* were determined. The relative mRNA expression was assessed using $2^{-\Delta\Delta Cq}$ method (10). Targeted prediction of *miR-122-5p* was analyzed by TargetScan (www.targetscan.org).

Western blotting. Anti-AFP (dilution 1:200; cat. no. ab54745), anti-bcl-2 (dilution 1:500; cat. no. ab692), anti-caspase-3 (dilution 1:5,000; cat. no. 32351) and anti- β -actin (dilution 1:1,000; cat. no. ab8227) were purchased from Abcam (Cambridge, MA, USA). The cells were harvested in RIPA buffer (Thermo Fisher Scientific, Inc.) supplemented with protease inhibitors (Cell Signaling Technology, Inc., Danvers, MA, USA) and the protein concentrations were determined using the bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc.). Cell lysate proteins (40 μ g/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto polyvinylidene fluoride (PVDF) membranes (Merck USA, Minneapolis, MN, USA). The PVDF membranes were then incubated in Odyssey blocking buffer (PBS) (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. The PVDF membranes were then incubated with primary antibodies overnight at 4°C. Then, the membranes were washed four times for 5 min in PBS and incubated with goat anti-rabbit and anti-mouse IgG conjugated to horseradish peroxidase (dilution 1:1,000; cat. nos. ab6721 and ab6789; Abcam) buffer for 1 h at room temperature. The membranes then were probed with the indicated antibodies, and proteins were detected by an ECL Prime Western Blotting Detection reagent (Thermo Fisher Scientific, Inc.).

Proliferation assay and cell cycle analysis. To assess cell growth, the number of viable cells at various time-points after transfection was assessed by the colorimetric water-soluble tetrazolium salt assay (Cell Counting Kit-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA). Cell viability was determined by reading the optical density at 450 nm. The cell cycle was evaluated 24 h after transfection by fluorescence-activated cell sorting (FACS) (BD Biosciences, Franklin Lakes, NJ, USA). For the FACS analysis, harvested cells were fixed in 70% cold ethanol and treated with RNase (Sigma-Aldrich; Merck KGaA) and propidium iodide (PI; Sigma-Aldrich; Merck KGaA).

Apoptotic cell analysis. The apoptotic cells were evaluated 24 h after transfection by FACS. Transfected cells were harvested and stained with fluorescein isothiocyanate FITC-conjugated Annexin V and phosphatidylinositol (PI) using an Annexin V kit (Beckman Coulter, Inc., Brea, CA, USA).

Statistical analysis. Statistical significance was determined using GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, CA, USA). Quantitative values were expressed as the means \pm standard deviation (SD) unless noted otherwise. Statistical significance was evaluated using Student's t-test and one-way analysis of variance (ANOVA) for each time-point, followed by Tukey's post hoc test. The correlation between *miR-122-5p* and *FOXO3* in GC tissue samples was analyzed by Pearson's correlation analysis. $P < 0.05$ was considered to indicate a statistically significant result.

Results

Association between AFP and *miR-122-5p* levels in GC cell lines. The expression levels of AFP and *miR-122-5p*

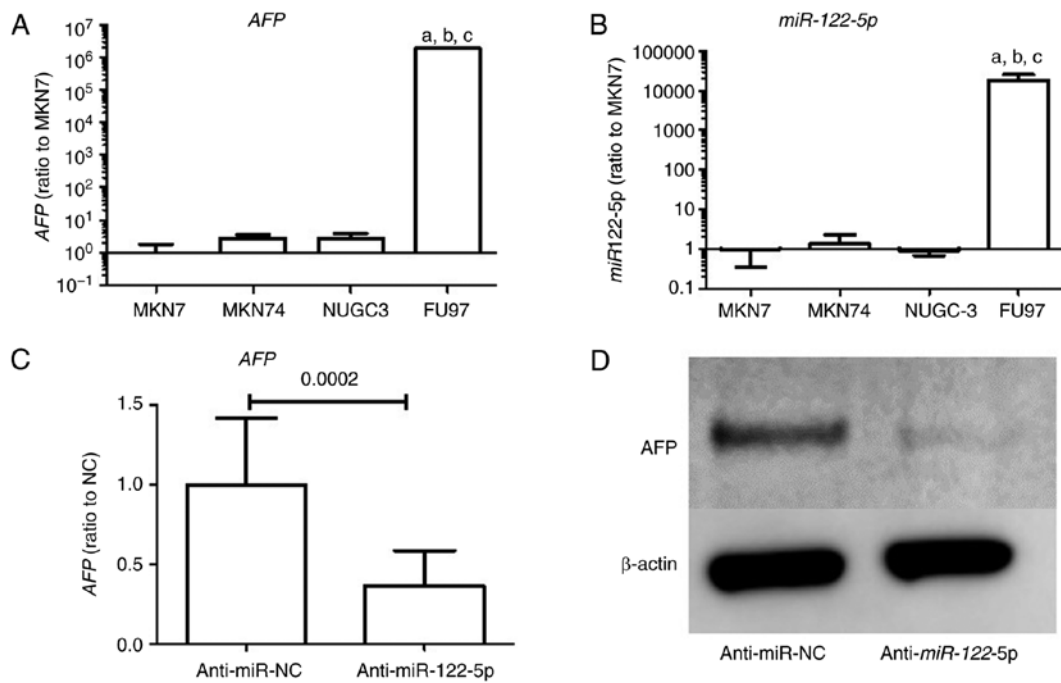


Figure 1. Relationship between AFP and *miR-122-5p* levels in GC cell lines. Quantification of (A) *AFP* levels and (B) *miR-122-5p* expression levels in GC cell lines by qRT-PCR. (C) Comparison of *AFP* levels between the negative control and anti-*miR-122-5p* in the FU97 cell line as determined by qRT-PCR. (D) Comparison of the protein expression of AFP between the negative control and anti-*miR-122-5p* in the FU97 cell line as determined by western blotting. * $P < 0.05$, compared to MKN7, ^b $P < 0.05$, compared to MKN74 and ^c $P < 0.05$, compared to NUGC-3. AFP, α ; GC, gastric cancer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

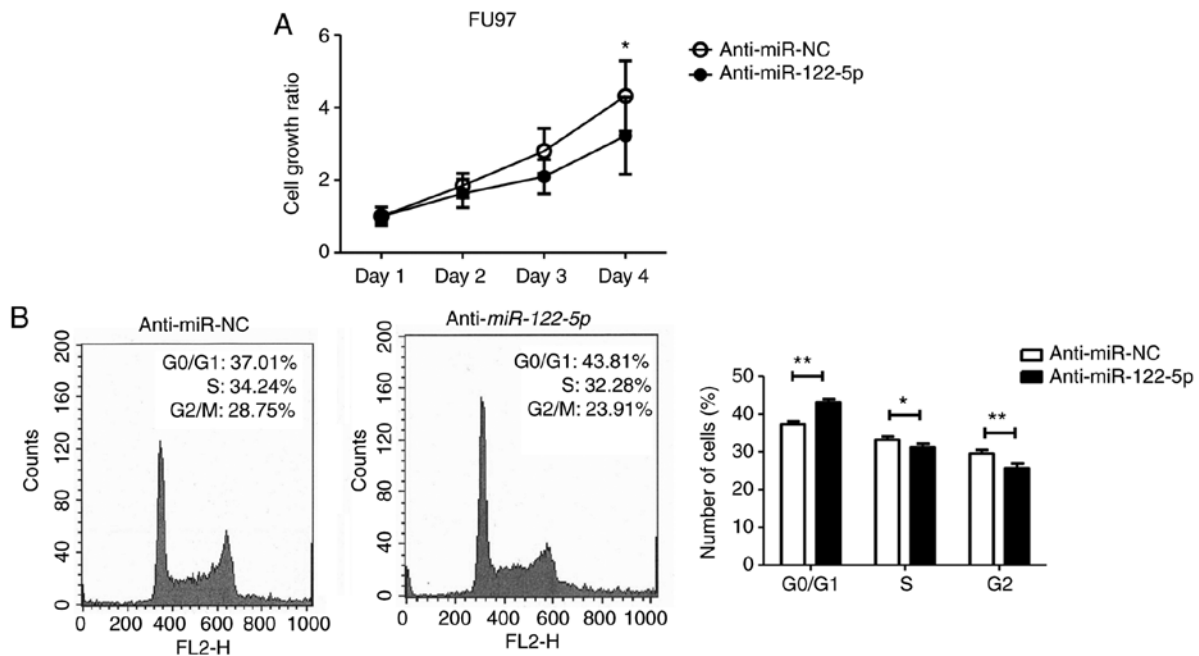


Figure 2. (A) Cell proliferation analysis and (B) FACS analysis in the FU97 cell line with inhibition of *miR-122-5p*. * $P < 0.05$, ** $P < 0.01$. FACS, fluorescence-activated cell sorting.

in GC cell lines were significantly higher in FU97 than in the other non-AFPGC cell lines as determined by qRT-PCR (Fig. 1A and B). Moreover, suppression of *miR-122-5p* reduced *AFP* levels in the FU97 cell line as determined by qRT-PCR (Fig. 1C). Protein expression of AFP was also decreased by *miR-122-5p* suppression in the FU97 cell line as determined by western blotting (Fig. 1D).

Investigation of the function of miR-122-5p in the AFPGC cell line. To investigate the function of *miR-122-5p* in AFPGC, we first performed a cell proliferation assay using *miR-122-5p* inhibitor. Proliferation of FU97 was significantly inhibited with *miR-122-5p* inhibitor transfection compared with negative control inhibitor transfection (Fig. 2A). FACS analysis revealed that inhibition of *miR-122-5p* induced G0/G1 cell cycle arrest (Fig. 2B).

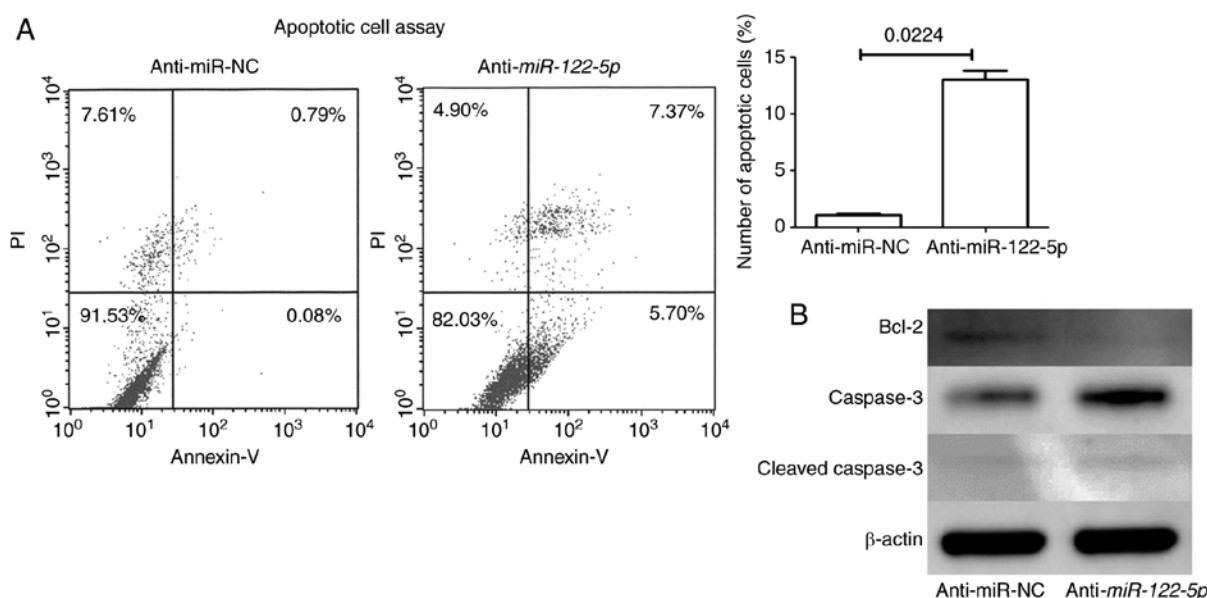


Figure 3. Apoptotic cell analysis. (A) Apoptotic cell analysis in the FU97 cell line with inhibition of *miR-122-5p*. (B) Comparison of the protein expression of Bcl-2, caspase-3 and cleaved caspase-3 between the negative control and anti-*miR-122-5p* in the FU97 cell line as determined by western blotting.

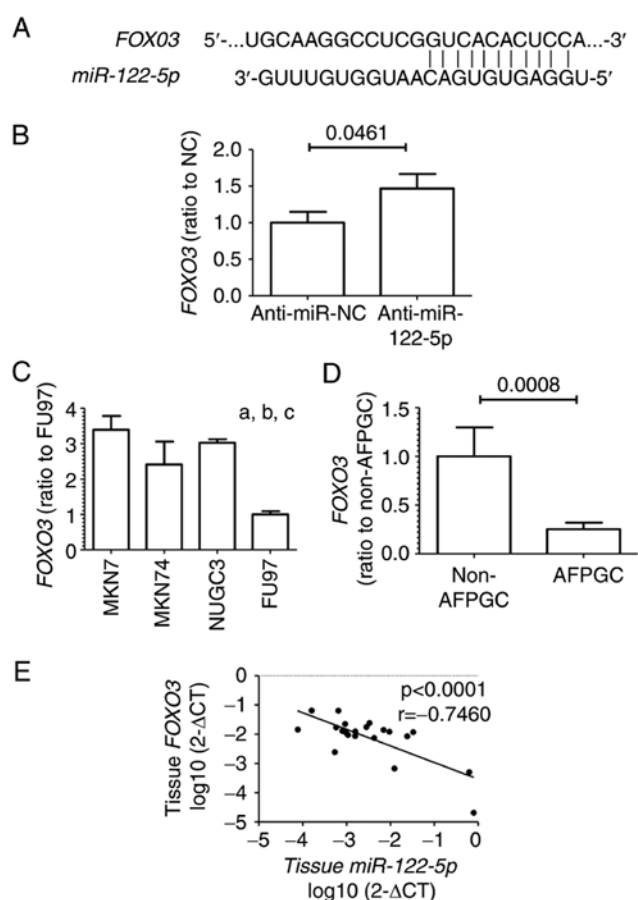


Figure 4. Relationship between *FOXO3* and *miR-122-5p* in GC cell lines and GC tissue samples. (A) The binding sites between *FOXO3* and *miR-122-5p*. (B) Comparison of *FOXO3* levels between the negative control and anti-*miR-122-5p* in the FU97 cell line by qRT-PCR. Quantification of *FOXO3* levels in (C) GC cell lines and (D) GC tissue samples by qRT-PCR. (E) Relationship between *FOXO3* levels and *miR-122-5p* expression levels in GC tissue samples. ^a $P < 0.05$, compared to MKN7; ^b $P < 0.05$, compared to MKN74 and ^c $P < 0.05$, compared to NUGC-3. AFPGC, α -fetoprotein producing gastric cancer; GC, gastric cancer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

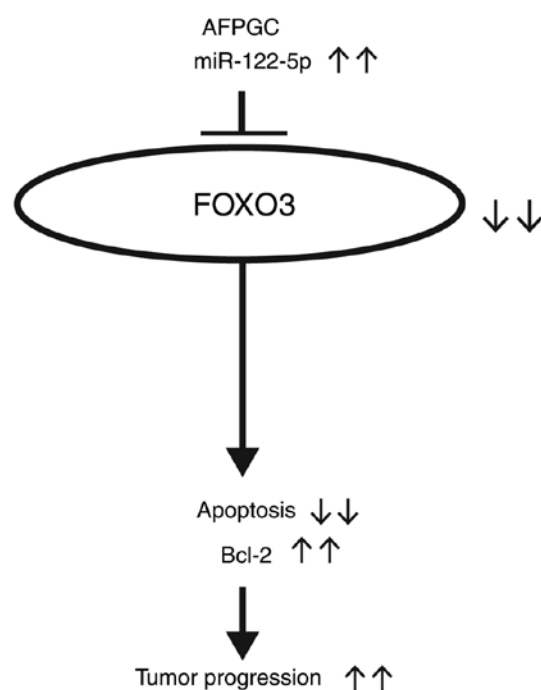


Figure 5. Schematic representation of the proposed role of *miR-122-5p* in AFPGC. *miR-122-5p* inhibits apoptosis and facilitates tumor progression by targeting *FOXO3*. AFPGC, α -fetoprotein-producing gastric cancer.

Next, apoptotic cell analysis revealed that inhibition of *miR-122-5p* increased early (Annexin V-positive/PI-negative) and late (Annexin V-positive/PI-positive) apoptosis (Fig. 3A). Western blotting revealed that the expression of anti-apoptotic protein (Bcl-2) was decreased and that of pro-apoptotic protein (caspase-3 and cleaved caspase-3) was increased with *miR-122-5p* inhibition in FU97 cells (Fig. 3B).

Association between *FOXO3* and *miR-122-5p* levels in GC cell lines and GC tissue samples. To further investigate the

molecular mechanism of *miR-122-5p* suppressing tumor progression in AFPGC, the miRNA target analysis tool (TargetScan; www.targetscan.org) was used to predict potential targets of *miR-122-5p*. Among numerous potential targets, we selected FOXO3, which is involved in cell cycle arrest and apoptosis, for further analyses in the present study (Fig. 4A). Inhibition of *miR-122-5p* increased FOXO3 levels in the FU97 cell line as determined by qRT-PCR (Fig. 4B). Moreover, among the GC cell lines, the expression levels of FOXO3 were significantly lower in the FU97 cells than in the other non-AFPGC cell lines as revealed by qRT-PCR (Fig. 4C).

We also investigated the FOXO3 expression in 25 GC tissue samples including 5 AFPGC patients. FOXO3 levels were significantly lower in AFPGC than in non-AFPGC tissue samples (Fig. 4D). Moreover, *miR-122-5p* expression levels were inversely correlated with FOXO3 levels ($r=-0.7460$, $P<0.0001$; Fig. 4E).

Discussion

Although α -fetoprotein-producing gastric cancer (AFPGC) has been recognized as an aggressive gastric cancer (11,12), to the best of our knowledge no comprehensive molecular analysis has been reported for this uncommon subtype. We previously identified that *miR-122-5p* was significantly highly expressed in AFPGC tissues and could be a useful plasma biomarker in patients (9). Moreover, we revealed that *miR-122-5p* exhibited a strong correlation with malignant potential in clinical settings (9). In the present study, we confirmed that the expression level of *miR-122-5p* was significantly increased in the AFPGC cell line than in common gastric cancer (GC) cell lines, and also inhibition of *miR-122-5p* significantly reduced AFP levels in the AFPGC cell line. Conversely, several studies have clearly demonstrated that the AFP molecule itself directly contributes to the promotion of tumor progression (13-15). With regard to these findings, we hypothesized that *miR-122-5p* may directly regulate tumor progression in AFPGC.

Current functional analyses by the gene transfection approach clearly demonstrated that inhibition of *miR-122-5p* significantly reduced proliferation in AFPGC cells through induction of apoptosis. Several previous studies have revealed that resistance to apoptosis could play a crucial role in the aggressive characteristics of AFPGC (14,15). Apoptotic resistance may be a major cause of the low chemosensitivity in AFPGC.

Next, we investigated the possible targets of *miR-122-5p* and identified FOXO3 as a likely candidate. FOXO3 is a critical protein involved in cell cycle arrest and apoptosis (16,17). Therefore, FOXO3 is recognized as a tumor suppressor, and decreased FOXO3 expression is associated with progression of various tumors, including GC (18-20). Guo *et al* (21) revealed that *miR-122-5p* downregulated FOXO3 expression by binding to the 3'UTR of its mRNA. A luciferase reporter assay was carried out by them, and it revealed direct interaction between *miR-122-5p* with FOXO3 (21). We also revealed that *miR-122-5p* overexpression inhibited FOXO3 by qRT-PCR and western blotting in GC cell line NUGC-3 (Fig. S1). We confirmed that suppression of *miR-122-5p* increased FOXO3 levels, and *miR-122-5p* expression levels were inversely correlated with

FOXO3 levels in GC tissue samples. Moreover, expression of FOXO3 was significantly lower in AFPGC than in non-AFPGC cell lines and clinical tumor tissues. Collectively, FOXO3 is an important target of *miR-122-5p* in AFPGC, which inhibits apoptosis and subsequently facilitates tumor progression (Fig. 5).

In several previous studies, *miR-122-5p* was down-regulated and functioned as a tumor suppressor in various cancers, such as hepatocellular (22), non-small cell lung (23), gallbladder (24), bladder (25) and breast cancer (26) as well as GC (27-29). However, we clearly revealed that *miR-122-5p* facilitated tumor progression in AFPGC cells and speculated that the function of *miR-122-5p* and mechanisms underlying tumor progression may be distinct between AFPGC and non-AFPGC.

Why AFPGC causes liver-specific metastasis, the most distinct clinical characteristic of the disease, remains unknown. In the present study, the migration and invasion abilities of FU97 were not significantly altered by inhibition of *miR-122-5p* (data not shown). Moreover, *miR-122-5p* was not correlated with liver metastasis of non-AFPGC in clinical settings (data not shown). He *et al* (30) revealed that upregulated Bcl-2 (anti-apoptotic protein) was correlated with liver metastasis of AFPGC in clinical settings. Similarly, the protein level of Bcl-2 was decreased by inhibition of *miR-122-5p* in the present study. We surmised that the anti-apoptotic effect may be important for the progression of liver metastasis in AFPGC. However, further studies are warranted to demonstrate the biological function of *miR-122-5p* for liver metastasis in AFPGC.

In conclusion, *miR-122-5p* inhibited apoptosis and facilitated tumor progression by targeting FOXO3 in AFPGC, which indicated the possibility of *miR-122-5p* as a potential therapeutic target in AFPGC.

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Availability of data and materials

Data were collected at the University of Yamanashi and are not publicly available.

Authors' contributions

SM performed the majority of the experiments and wrote the manuscript; SF and RS performed the research; KS, HAK and YK provided the tissue samples and the clinical data, and reviewed and edited the manuscript; HS, NH, HAM, HKA, MS, SI and HKO made substantial contributions to the data analysis and interpretation. DI designed the study and helped to draft the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Yamanashi University (approval no. 1825) and was performed in accordance with the ethical standards of the Declaration of Helsinki and its amendments. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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