

LncRNA-AWPPH activates TGF- β 1 in colorectal adenocarcinoma

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Abstract. Long non-coding RNA associated with poor prognosis of hepatocellular carcinoma (lncRNA-AWPPH) is a newly discovered lncRNA that has important functions in the pathogenesis of several malignancies. However, its role in the development of colorectal adenocarcinoma is unknown. The current study therefore investigated the function of AWPPH in colorectal adenocarcinoma. A total of 86 patients with colorectal adenocarcinoma and 56 healthy controls were included. Tumor tissues and adjacent healthy tissues were collected from patients with colorectal adenocarcinoma, and blood was collected from both patients and healthy controls. Expression of AWPPH in tissues and blood was detected by the reverse transcription-quantitative polymerase chain reaction. Receiver operating characteristic curve analysis was used to evaluate the diagnostic value of serum AWPPH for colorectal adenocarcinoma. All patients were followed up for 5 years, and survival curve analysis was performed to investigate the association between serum level of AWPPH and patients' survival. The effects of AWPPH overexpression and silencing in colorectal adenocarcinoma cell lines were investigated. Effects on cell proliferation and viability were detected by the cell counting kit-8 and MTT assays, respectively. Effects on transforming growth factor β 1 (TGF- β 1) expression were determined by western blotting. AWPPH was significantly upregulated in tumor tissues compared with adjacent healthy tissues. AWPPH expression levels in blood increased in patients with colorectal adenocarcinoma compared with healthy controls, suggesting that AWPPH may be a sensitive and accurate diagnostic and prognostic biomarker for colorectal adenocarcinoma. AWPPH overexpression in colorectal adenocarcinoma cell lines promoted cell proliferation and increased cell viability, while AWPPH silencing resulted in opposite effects. AWPPH overexpression promoted and silencing inhibited TGF- β 1 expression. Therefore, lncRNA-AWPPH promoted colorectal adenocarcinoma by promoting tumor

growth, increasing tumor cell viability and activating the TGF- β 1 signaling.

Introduction

Colorectal cancer is the most common type of gastrointestinal malignancy and is the third most commonly diagnosed cancer and fourth leading cause of cancer-associated mortality worldwide (1). Colorectal adenocarcinoma is the most common cause of colorectal cancer, with 1.2 million new cases and 0.6 million mortalities per year worldwide (2). Surgical resection is the main treatment of primary colorectal adenocarcinoma. Approximately 90% of patients with colorectal adenocarcinoma live >5 years following appropriate surgical intervention. However, the 5-year survival rate for those with distant tumor metastasis remains as low as 10% even after detection of lymph node metastases (3). Therefore, early diagnosis and treatment is key for the survival of patients with colorectal adenocarcinoma.

Transforming growth factor β (TGF- β) binding to the cell surface triggers activation of multiple signal transduction pathways that are connected in intricate ways with each other, and with other response networks involved in sensing cellular information input. Recent data have indicated that changes in the signal intensity and connectivity of these pathways may underlie the complex transition of the TGF- β pathway from tumor suppressor to oncogene during tumorigenesis (4). TGF- β and its signaling effectors act as key determinants of carcinoma cell behavior. The autocrine and paracrine effects of TGF- β on tumor cells and the tumor micro-environment exert both positive and negative influences on cancer development (5). Accordingly, the TGF- β signaling pathway has been considered as both a tumor suppressor pathway and a promoter of tumor progression and invasion.

Besides messenger RNAs that encode protein products, the human genome also encodes a vast population of non-coding RNAs that have critical functions in both normal physiological processes and pathological changes (6). Long non-coding RNAs (lncRNAs) are a subgroup of non-coding RNAs composed of >200 nucleotides and have been revealed to serve important roles in the pathogenesis of human diseases including various types of malignancies (7,8). Long non-coding RNA associated with poor prognosis of hepatocellular carcinoma (lncRNA-AWPPH) is newly discovered lncRNA that has been shown to be involved in the pathogenesis of hepatocellular carcinoma (9) and bladder cancer (10). However, the function of lncRNA-AWPPH in other human diseases as well

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as in normal physiological processes is unknown. In this study, lncRNA-AWPPH promoted the growth of colorectal adenocarcinoma and was shown to have diagnostic and prognostic value in the disease.

Materials and methods

Subjects. A total of 86 patients with colorectal adenocarcinoma diagnosed via pathological examination were selected at the Qingdao Central Hospital (Qingdao, China) between January 2012 and January 2013. The patients included 49 males and 37 females, and the age ranged between 26 to 72 years, with a mean age of 50.2 ± 7.2 years. The inclusion criteria were as follows: i) Pathologically diagnosed as colorectal adenocarcinoma; ii) patients signed informed consent; and iii) patients willing to cooperate with researchers. The exclusion criteria were as follows: i) patients with other types of malignant tumors; ii) patients with severe coagulation dysfunction; and iii) patients with other colorectal diseases. There were 10 patients in American Joint Committee on Cancer stage II, 16 in stage III and 60 in stage IV. At the same time, a total of 56 healthy individuals were selected at the Qingdao Central Hospital (Qingdao, China) between January 2012 and January 2013 to serve as a control group. The patients included 29 males and 27 females, with a mean age of 51.2 ± 6.4 years (range, 26-72 years). The inclusion criteria were as follows: i) Healthy individuals were not diagnosed pathologically with colorectal adenocarcinoma; ii) healthy individuals provided written informed consent; and iii) healthy individuals willing to cooperate with researchers. The exclusion criteria were as follows: i) Individuals with other types of malignant tumors; ii) healthy individuals with severe coagulation dysfunction; and iii) healthy individuals with other colorectal diseases. All participants were Han Chinese. All participants signed informed consent and the present study was approved by the Ethics Committee of Qingdao Central Hospital. After discharge, patients were followed up for 5 years (until January 2018) or until their mortality.

Specimen collection. Whole blood (20 ml) was obtained from the 86 patients with colorectal adenocarcinoma and the 56 healthy individuals on the day of admission. Blood samples were kept at room temperature for 1.5 h, followed by centrifugation at $1,800 \times g$ for 20 min to collect serum. All patients received surgical resection of the primary tumors, and tumor tissues and adjacent healthy tissues within 5 cm of the tumor were collected from 3 different sites and preserved in liquid nitrogen before use. All tissue samples were confirmed by pathological examination.

Cell lines and cell culture. *Homo sapiens* colorectal adenocarcinoma cell lines HT-29 [Caucasian American Type Culture Collection (ATCC)-formulated McCoy's 5a Medium Modified; cat. no. 30-2007 add 10% fetal bovine serum, ATCC 30-2020 and 1% Penicillin-Streptomycin, GBICO15140-22], Hs 698.T (American Indian; ATCC-formulated Dulbecco's Modified Eagle's Medium; cat. no. 30-2002 add 10% fetal bovine serum, ATCC 30-2020 and 1% Penicillin-Streptomycin, GBICO15140-22) and SNU-C1 (Asian; add 10% fetal bovine serum; ATCC 30-2020 and 1% Penicillin-Streptomycin;

GBICO15140-22) were purchased from the American Type Culture Collection. Cells were cultured under the conditions recommended by the ATCC in an incubator at 37°C and 5% CO_2 . Cells were harvested during logarithmic growth phase for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Tumor tissues and adjacent healthy tissues were ground in liquid nitrogen. TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) was used to extract total RNA by mixing directly with *in vitro* cultured cells. SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific, Inc.) was used to reverse transcribe total RNA into cDNA. Reverse transcription conditions were: 10 min at 25°C , 35 min at 50°C and 15 min at 80°C . SYBR[®] Green Real-Time PCR Master Mix (Thermo Fisher Scientific, Inc.) was used to perform RT-qPCR using a CFX96 Touch[™] Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The following primer pairs were used: 5'-CTGGATGGTTCGCTGCTTTTAA-3' (forward) and 5'-AGG GGGATGAGTCGTGATTT-3' (reverse) for lncRNA-AWPPH; 5'-CCCACTCCTCCACCTTTGAC-3' (forward) and 5'-ATG AGGTCCACCACCCTGTT-3' (reverse) for human GAPDH. The following thermocycling conditions were used: 95°C for 15 sec, followed by 40 cycles of 95°C for 14 sec and 50°C for 42 sec. The $2^{-\Delta\Delta\text{C}_q}$ method was used to process all data (11), and relative expression level of lncRNA-AWPPH was normalized to endogenous control GAPDH.

Construction of the lncRNA AWPPH vector and silencing cell lines. A lncRNA-AWPPH expression vector was constructed by inserting an *EcoRI-EcoRI* fragment containing full length lncRNA-AWPPH cDNA into a pIRES2-EGFP vector (Clontech Laboratories, Inc.). Negative control short hairpin (shRNA) targeting sequence 5'-GACTTCATAAGGCGCATG C-3' and shRNA lncRNA-AWPPH targeting sequence 5'-GGA ATGCAGCTGAAAGATTCC-3' were synthesized by Chang Jing Bio-Tech, Ltd. Lipofectamine[®] 2000 (cat. no. 11668-019; Invitrogen; Thermo Fisher Scientific, Inc.) was used to transfect 10 nM vector or 50 mM shRNA into 5×10^6 cells for 4 h. Transfections with empty pIRES2-EGFP vector or negative control shRNA were used as negative controls. The control group cells were untransfected.

Cell proliferation assay. A total of 4×10^4 cells in 100 μl cell suspension were added into each well of 96-well plates. Cells were cultured in an incubator (37°C , 5% CO_2), and 10 μl Cell Counting Kit-8 solution (Beijing Solarbio Science & Technology Co., Ltd.; cat. no. CA1210-100) was added into each well 24, 48, 72 and 96 h later. Cells were cultured at 37°C for another 4 h, and optical density (OD) values at 450 nm were measured using an accuSkan[™] GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The sample with the largest OD value was set to 100 and all cell proliferation values were normalized to this sample.

MTT assay. The cell suspension was diluted using culture medium (ATCC) to obtain a final cell density of 4×10^4 cells/ml. A total of 10 mM tetraethylammonium was then added to the cell suspension. A total of 4×10^3 cells in 100 μl cell suspension were added into each well of a 96-well plate. Cells were allowed

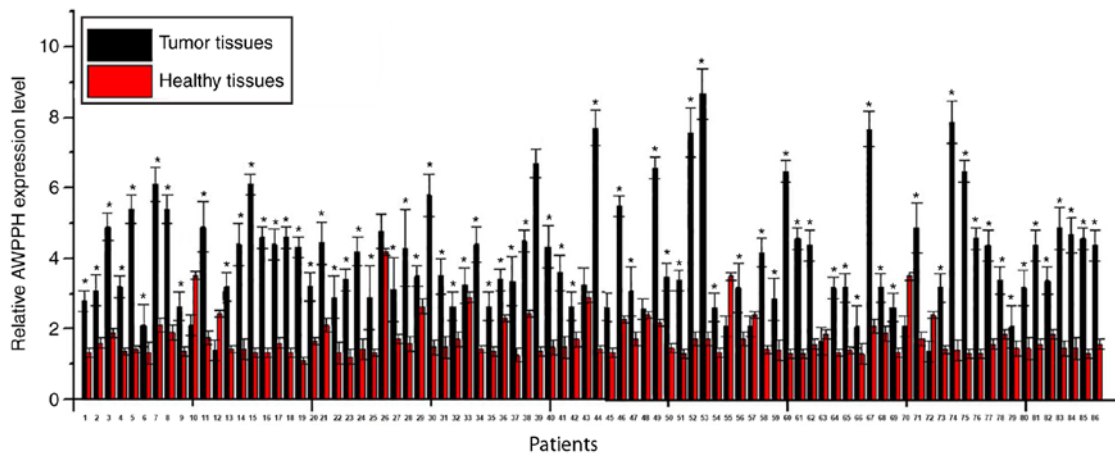


Figure 1. Expression level of lncRNA-AWPPH in tumor tissues and adjacent healthy tissues of patients with colorectal adenocarcinoma. Data are presented as the mean values for tumor and healthy adjacent tissue samples collected from 3 different sites. * $P < 0.05$ vs. adjacent healthy tissues. lncRNA-AWPPH, long non-coding RNA associated with poor prognosis of hepatocellular carcinoma.

to incubate at 37°C and $5\% \text{CO}_2$ for 6 h. This was followed by a 4-h incubation period with $10 \mu\text{l}$ MTT (DMSO). Absorbance at a wavelength of 570 nm was measured using an accuSkan™ GO UV/Vis microplate spectrophotometer (Thermo Fisher Scientific, Inc.). The percentage of viable cells was calculated according to the following formula: $\% \text{ cell viability} = (\text{absorbance sample} - \text{absorbance blank}) / (\text{absorbance control} - \text{absorbance blank}) \times 100$. The sample with the highest cell viability was set to 100 and all other samples were normalized to this sample.

Western blot analysis. Total protein was extracted from *in vitro* cultured cells using radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). Total protein was quantified using a bicinchoninic acid assay. Following boiling at 100°C for 5 min for the denaturation, $20 \mu\text{g}$ protein/lane was separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto a polyvinylidene fluoride membrane (Thermo Fisher Scientific, Inc.). Membranes were washed with TBST buffer 3 times, 15 min each time. The membranes were incubated with primary antibodies TGF- $\beta 1$ (1:2,000; cat. no. ab92486; Abcam) and GAPDH (1:2,000; cat. no. ab181602; Abcam) on the shaker at 4°C overnight. Membranes were washed with TBST buffer 3 times, 15 min each time. Membranes were incubated with a horseradish peroxidase-labeled secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource) for 2 h at room temperature. Membranes were washed with TBST buffer 3 times, 15 min each time. The chemiluminescence method was used to detect the protein bands (EMD Millipore). Relative expression level of TGF- $\beta 1$ was normalized to the endogenous control GAPDH using Image J software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All statistical analyses were performed using GraphPad Prism Software (version 6; GraphPad Software, Inc.). Gene expression and cell proliferation and viability data are presented as the mean \pm standard deviation. Comparisons between two groups were performed using a paired or unpaired Student's t-test, as appropriate, while comparisons between multiple groups were performed using the one-way analysis of variance followed by the Least Significant Difference test.

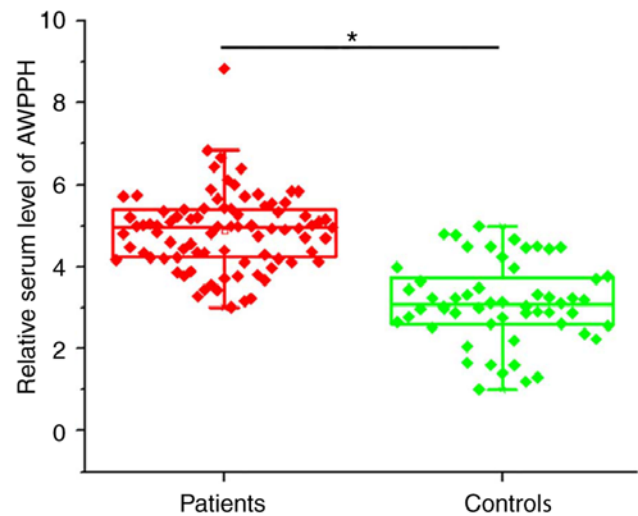


Figure 2. Expression of lncRNA-AWPPH in serum of healthy individuals and patients with colorectal adenocarcinoma. * $P < 0.05$. lncRNA-AWPPH, long non-coding RNA associated with poor prognosis of hepatocellular carcinoma.

Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic value of serum AWPPH for colorectal adenocarcinoma, with patients with colorectal adenocarcinoma as true positive cases and healthy controls as true negative cases. The 86 patients with colorectal adenocarcinoma were divided into high expression group ($n=43$) and low expression group ($n=43$) according to the median serum level of AWPPH. The Kaplan-Meier method was used to plot survival curves for both groups, and survival curves were compared by log-rank test. Figures were plotted using Origin software (version 9.1; OriginLab, Northampton, MA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of lncRNA-AWPPH is significantly higher in tumor tissues compared with adjacent healthy tissues in the majority

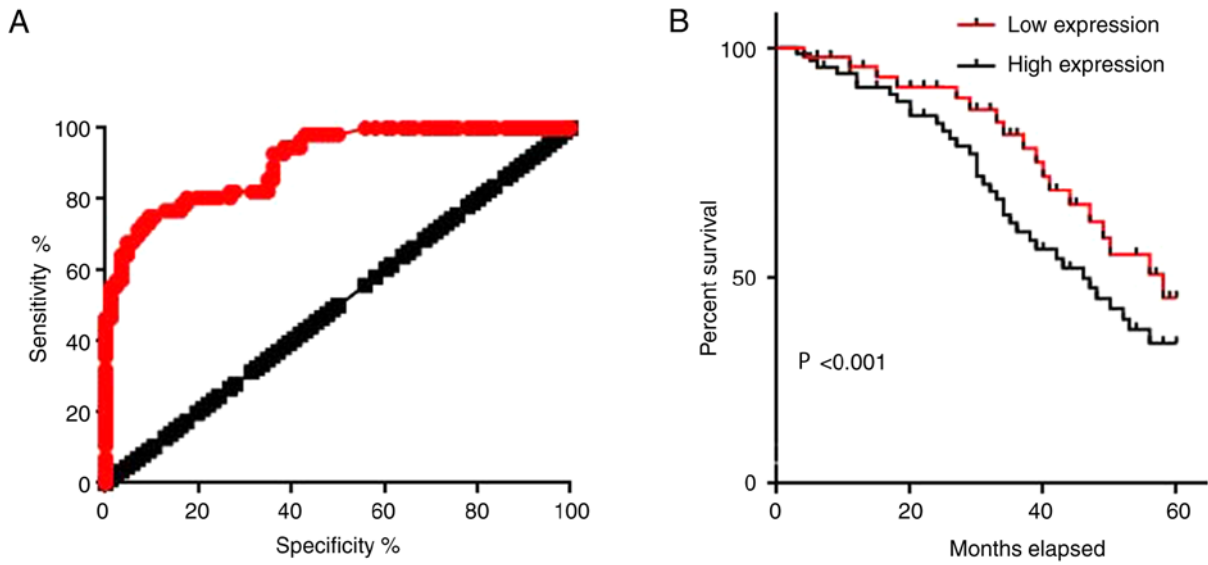


Figure 3. Diagnostic and prognostic values of serum lncRNA-AWPPH for colorectal adenocarcinoma. (A) Receiver operating characteristic curve analysis of the diagnostic value of serum lncRNA-AWPPH for colorectal adenocarcinoma. (B) Comparison of survival curves of patients with high and low serum levels of serum lncRNA-AWPPH to evaluate the prognostic value of serum lncRNA-AWPPH for colorectal adenocarcinoma. lncRNA-AWPPH, long non-coding RNA associated with poor prognosis of hepatocellular carcinoma.

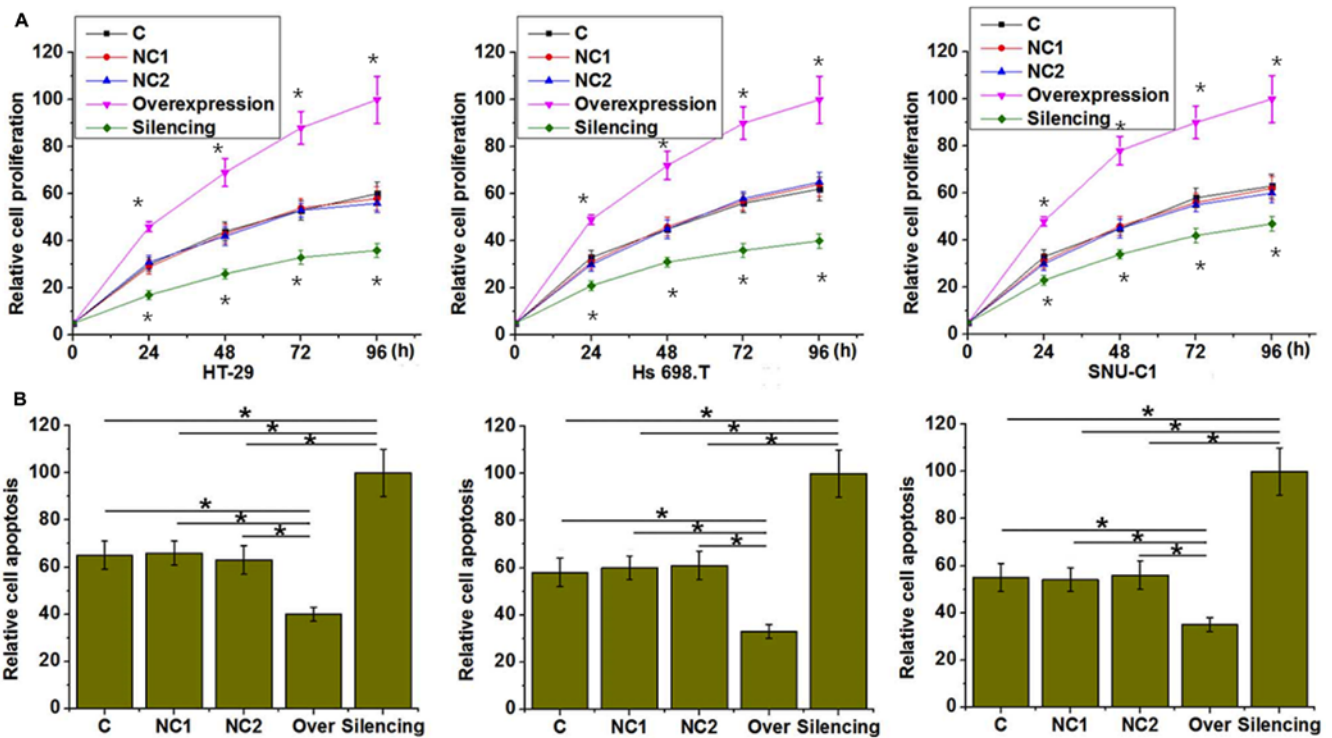


Figure 4. Effects of lncRNA-AWPPH overexpression and silencing on the proliferation and viability of colorectal adenocarcinoma cell lines. (A) Effects of lncRNA-AWPPH overexpression and silencing on colorectal adenocarcinoma cell proliferation at 24, 48, 72 and 96 h. *P<0.05. (B) Effects of lncRNA-AWPPH overexpression and silencing on colorectal adenocarcinoma cell viability. *P<0.05. NC1, negative control 1 (transfection with negative control shRNA); NC2, negative control 2 (transfection with empty pIRES2-EGFP vector); over, overexpression; lncRNA-AWPPH, long non-coding RNA associated with poor prognosis of hepatocellular carcinoma; C, untransfected control group.

of patients with colorectal adenocarcinoma. Expression levels of lncRNA-AWPPH in tumor tissues and adjacent healthy tissues (collected from 3 different sites) of 86 patients with colorectal adenocarcinoma were detected by RT-qPCR. A significantly higher expression level of lncRNA-AWPPH was observed in tumor tissues compared with adjacent healthy

tissues in 75 out of 86 patients, accounting for 87.2% (Fig. 1; all P<0.05). By contrast, a significantly lower expression level of lncRNA-AWPPH in tumor tissues compared to adjacent healthy tissues was found in 6 out of 86 patients, accounting for 7%. No significant difference was found in 5 cases, accounting for 5.8%. These data suggested that upregulation of

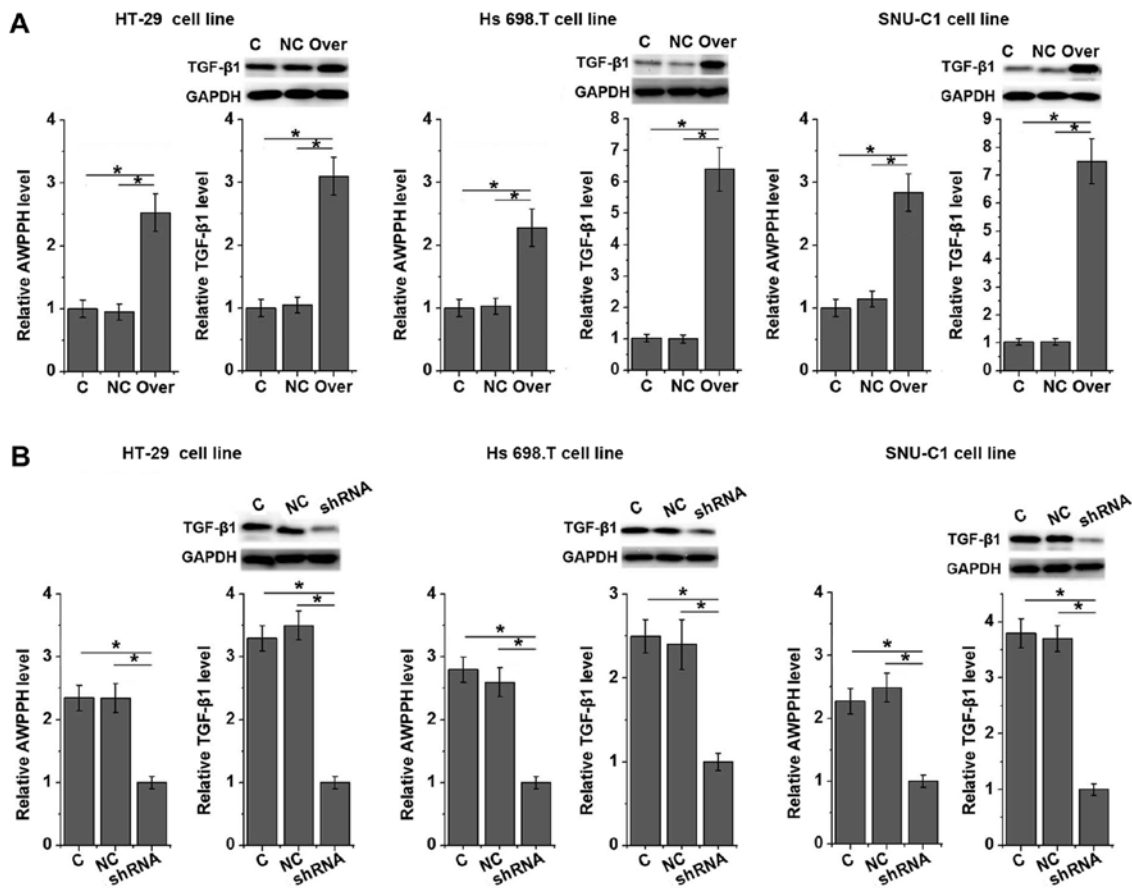


Figure 5. Effects of lncRNA-AWPPH overexpression and silencing on TGF- β 1 expression. (A) Effects of lncRNA-AWPPH overexpression on TGF- β 1 expression. (B) Effects of lncRNA-AWPPH silencing on TGF- β 1 expression. * $P < 0.05$. lncRNA-AWPPH, long non-coding RNA associated with poor prognosis of hepatocellular carcinoma; TGF- β 1, transforming growth factor β ; shRNA, short hairpin RNA; C, untransfected control group; NC, negative control (transfections with empty pIRES2-EGFP vector or negative control shRNA); over, overexpression.

lncRNA-AWPPH is likely to be involved in the pathogenesis of colorectal adenocarcinoma.

Expression of lncRNA-AWPPH in serum is higher in patients with colorectal adenocarcinoma. Compared with healthy controls, expression of lncRNA-AWPPH in the serum of the 56 healthy individuals and the 86 patients with colorectal adenocarcinoma was also detected by RT-qPCR. Serum levels of lncRNA-AWPPH were significantly higher in patients with colorectal adenocarcinoma compared with healthy controls ($P < 0.05$; Fig. 2).

Serum lncRNA-AWPPH has a diagnostic and prognostic value for colorectal adenocarcinoma. ROC curve analysis was performed to evaluate the diagnostic value of serum lncRNA-AWPPH for colorectal adenocarcinoma. The area under the curve was 0.9065, with a 95% confidence interval of 0.8590-0.9539 ($P < 0.0001$; Fig. 3A). The 86 patients with colorectal adenocarcinoma were divided into a high expression group ($n=43$) and a low expression group ($n=43$) according to the median serum level of lncRNA-AWPPH. The Kaplan-Meier method was used to plot survival curves for both groups, and the curves were compared by log rank test. Results revealed that the overall survival of patients with a high serum level of lncRNA-AWPPH was significantly worse than that of patients with a low serum level of lncRNA-AWPPH ($P < 0.001$; Fig. 3B). These data suggested that serum lncRNA-AWPPH

may serve as a promising diagnostic and prognostic biomarker for colorectal adenocarcinoma.

Effects of lncRNA-AWPPH overexpression and silencing on colorectal adenocarcinoma cell proliferation and viability. In the current study, three colorectal adenocarcinoma cell lines derived from patients with different ethnic backgrounds, namely HT-29 (Caucasian), Hs 698.T (American Indian) and SNU-C1 (Asian), were investigated (Fig. 4). lncRNA-AWPPH overexpression and silencing in the cell lines were confirmed by RT-qPCR (Fig. 5). lncRNA-AWPPH overexpression and silencing promoted and inhibited the proliferation of colorectal adenocarcinoma cells respectively, compared with the cells transfected with negative control constructs (Fig. 4A). In addition, lncRNA-AWPPH overexpression and silencing increased and decreased the viability of colorectal adenocarcinoma cells respectively, compared with their respective control groups.

Effects of lncRNA-AWPPH overexpression and silencing on TGF- β 1 expression. TGF- β 1 has been shown to promote the growth of colorectal adenocarcinoma (12). Therefore the effects of AWPPH overexpression and silencing on TGF- β 1 expression were investigated in the current study. AWPPH overexpression promoted (Fig. 5A) and silencing inhibited (Fig. 5B) TGF- β 1 expression, suggesting that AWPPH may upregulate TGF- β 1 expression in colorectal adenocarcinoma.

Discussion

Several factors are involved in the growth, development and progression of colorectal adenocarcinoma (13). lncRNAs have been implicated in the pathogenesis of colorectal adenocarcinoma (14), however, their exact roles have not been well studied. Colon cancer associated transcript 1 was found to be specifically expressed in colorectal adenocarcinoma tissues but not in normal tissue, and promoted the progression of tumor through multiple pathways (15). lncRNA-AWPPH is a recently discovered lncRNA that is upregulated during the development of hepatocellular carcinoma (9) and bladder cancer (10). The involvement of lncRNA-AWPPH in other human diseases as well as in normal physiological processes is unknown. In the present study, lncRNA-AWPPH was found to be significantly upregulated in colorectal adenocarcinoma compared with adjacent healthy tissues in the majority of patients with colorectal adenocarcinoma. In addition, the expression level of lncRNA-AWPPH in serum was significantly increased in patients with colorectal adenocarcinoma compared with healthy controls. These data suggested that upregulation of lncRNA-AWPPH is likely to be involved in the pathogenesis of colorectal adenocarcinoma.

The prognosis of patients with colorectal adenocarcinoma is poor (3) and early diagnosis and treatment is critical. The development of human diseases may be accompanied by changes in blood composition, and the detection of specific substances may be of diagnostic value (16,17). In the current study, ROC curve analysis revealed that serum lncRNA-AWPPH can be used to sensitively and effectively distinguish patients with colorectal adenocarcinoma from normal controls. In addition, higher serum level of lncRNA-AWPPH predicted shorter survival time. Taken together, these data suggested that serum lncRNA-AWPPH may serve as a promising diagnostic and prognostic biomarker for colorectal adenocarcinoma. lncRNA-AWPPH expression may be affected by multiple malignancies (9,10), which may affect the specificity of serum lncRNA-AWPPH as a diagnostic and prognostic marker of colorectal adenocarcinoma. Therefore, multiple biomarkers should be combined to increase specificity.

It is known that ethnic backgrounds affect the pathogenesis of different types of malignancies (18-20). In the present study, lncRNA-AWPPH was shown to promote the proliferation and increase the viability of three colorectal adenocarcinoma cell lines derived from patients with different ethnic backgrounds. Furthermore, it has been reported that lncRNA-AWPPH promoted the proliferation of cells in both hepatocellular carcinoma (9) and bladder cancer (10).

The TGF- β 1 signaling has been implicated in the growth and metastasis of colorectal adenocarcinoma (12,21,22). In the current study, lncRNA-AWPPH overexpression and silencing significantly promoted and inhibited the expression of TGF- β 1 protein in all three colorectal adenocarcinoma cell lines respectively. Cancer cell stemness is critical for cancer progression (23) and the TGF- β signaling serves an important role in the maintenance of cancer cell stemness (24). Therefore, lncRNA-AWPPH may interact with the TGF- β signaling to participate in the biological behaviors of cancer stem cells in colorectal adenocarcinoma.

In conclusion, AWPPH expression was significantly upregulated in tumor tissues compared with adjacent healthy tissues in the majority of patients with colorectal adenocarcinoma in the current study. AWPPH expression level in blood was increased in patients with colorectal adenocarcinoma compared with healthy controls. Blood AWPPH may therefore serve as a promising diagnostic and prognostic biomarker for colorectal adenocarcinoma. AWPPH increased cancer cell proliferation and viability as well as TGF- β 1 expression in colorectal adenocarcinoma in the present study. Therefore, lncRNA-AWPPH can promote the growth of colorectal adenocarcinoma by promoting tumor growth, increasing tumor cell viability and activating the TGF- β 1 signaling.

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Availability of data and materials

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

Authors' contributions

CL and CY conceived and designed the study. CL and BH performed experiments and analyzed the data. JX interpreted the data. CY drafted the manuscript and all authors approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Qingdao Central Hospital (Qingdao, China) and informed consent was obtained.

Patient consent for publication

All patients signed informed consent.

Competing interests

The authors declare that they have no competing interests.

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