MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2019; 25: 2535-2541
DOI: 10.12659/MSM.912876

Received: 2018.08.23
Accepted: 2018.12.08
Published: 2019.04.06

Long Non-Coding RNA AWPPH Promotes Postoperative Distant Recurrence in Resected Non-Small Cell Lung Cancer by Upregulating Transforming Growth Factor beta 1 (TGF-β1)

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Source of support: Departmental sources

Background: Postoperative recurrence of cancers is responsible for a large portion of deaths in cancer patients. Our study investigated the involvement of lncRNA AWPPH in recurrence of resected non-small cell lung cancer (NSCLC).

Material/Methods: A total of 128 patients were followed up for 3 years. Blood was extracted from each patient on the day of discharge, the day of the diagnosis of recurrence, or at the end of follow-up. Blood from 30 healthy controls was used as a control group. Patient were divided into 3 groups – a non-recurrence group (NR, n=54), a local recurrence group (LR, n=42), and a distant recurrence (DR, n=32) group – according to the follow-up results. Blood AWPPP was detected by qRT-PCR. AWPPH expression vectors were transfected into cells of human NSCLC cell lines. Cell migration and invasion were detected by Transwell migration and invasion assay, respectively. TGF-β1 expression was detected by Western blot analysis.

Results: Blood AWPPH levels were the highest in the DR group, followed by the LR and NR groups. The lowest blood AWPPH levels were observed in the control group. Blood AWPPH levels increased significantly in the DR group but not in the NR and LR groups during follow-up. Blood AWPPH levels were positively correlated with TGF-β1 mRNA levels in the DR group but not in the NR and LR groups during follow-up. AWPPH overexpression promoted cell migration and invasion and upregulated TGF-β1 expression.

Conclusions: lncRNA AWPPH can promote postoperative distant recurrence in resected NSCLC by upregulating TGF-β1.

MeSH Keywords: Carcinoma, Non-Small-Cell Lung • Recurrence • RNA, Long Noncoding • Transforming Growth Factor beta1

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/912876

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Background

Postoperative recurrence is responsible for a considerable portion of deaths among cancer patients [1]. Lung cancer is the most frequently diagnosed and most deadly type of cancer worldwide, which affects 1.35 million new cases, leading to 1.18 million cancer-related deaths each year [2]. Non-small cell lung cancer (NSCLC) is a major type of lung cancer, which is a serious public health problem in developing countries such as China [3]. More than half of patients with NSCLC develop NSCLC recurrence after surgical resection, and the survival rate of these patients is generally poor [4]. Postoperative recurrence in cancer patients includes 2 types: local and distant recurrence [5]. Compared with patients with local recurrence, patients who suffer from distant recurrence usually show even worse survival.

TGF-β has 2 opposite roles in cancer development [6]: it inhibits tumor growth in the early stages [7], but it promotes tumor metastasis in the late stages [8]. However, the involvement of TGF-β signaling in cancer postoperative recurrence still remains largely unknown. TGF-β signaling under certain conditions achieves its roles through interactions with long non-coding RNAs (lncRNAs) [9], which is a subgroup of non-coding RNAs composed of more than 200 nucleotides and has critical functions in both normal physical processes and pathological changes [10]. AWPPH is a recently identified lncRNA with an oncogenic role in hepatocellular carcinoma [11] and bladder cancer [12]. Our study found that AWPPH can upregulate TGF-β1 to promote postoperative distant recurrence of NSCLC.

Material and Methods

Patients, follow-up, and grouping

The clinicopathological findings of the patients are shown in Table 1. Our study included 128 patients pathologically diagnosed with NSCLC. These patients were treated in the Second Affiliated Hospital of Anhui Medical University from January 2012 to January 2015. Inclusion criteria were: 1) diagnosis and treatment were performed for the first time; 2) functions of other major organs were normal; 3) patients received surgical resection and completed 3-year follow-up; 4) patients were willing to participate. Exclusion criteria were: 1) patients who had other cancers; 2) patients who had other lung diseases; 3) patients who died before the diagnosis of recurrence; 4) patients older than 70 years on the day of discharge (due to the high prevalence of other diseases, such as chronic diseases among patients of this age group). For histological type, there were 71 cases of adeno-Ca, 46 cases of squamous-Ca, and 11 cases of adenosquamous-Ca. For nodal involvement, there were 46 cases of N0, 52 cases of N1, 24 cases of N2, and 6 cases of NX. For tumor stage, there were 46 cases of stage 1, 38 cases of stage 2, 28 cases of stage 3, and 16 cases of stage 4. For histological grade, there were 8 cases of G1, 56 cases of G2, and 64 cases of G3. Patients were divided into 3 groups – a non-recurrence group (NR, n=54), a local recurrence group (LR, n=42), and a distant recurrence group (DR, n=32) – according to the follow-up results. At the same time, 30 healthy people were also included to serve as a control group. See Table 1 for basic information of all subjects. No significant differences were found among the 4 groups of subjects in terms of age or gender. No significant differences in histological type, nodal involvement, tumor stage, or histological grade were found among patient groups.

Blood (10 ml) was extracted from the elbow vein of each participant on the day of discharge (all patients, n=126), the day of the diagnosis of recurrence (patients in LR and DR group, n=74), or at the end of follow-up (NR group, n=54). The Ethics Committee of the Second Affiliated Hospital of Anhui Medical University approved this study. All participants signed information consent.

Patients received different surgical treatments, such as segmentectomy, lobectomy, node dissection, and partial resections, depending on their clinical stages.

Real-time quantitative PCR (qRT-PCR)

Total RNA was extracted using Trizol reagent (Thermo Fisher Scientific) in strict accordance with instructions of the kit. cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to following conditions: 25°C for 5 min, 50°C for 25 min, and 80°C for 10 min. PCR reaction were performd using SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the following primers: 5'-CTGGATGGTCGCTGCTTTTTA-3' (forward) and 5'-AGGGGGATGAGTCGTGATTT-3' (reverse) for human lncRNA AWPPH; 5'-GACCTCTATGCCAACACAGT3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA3' (reverse) for β-actin. TGF-β1 primers were purchased from SinoBiological (Cat: HP100717). PCR parameters were: 95°C for 40 s, followed by 40 cycles of 95°C for 15 s, and 57.5°C for 20 s. $2^{-\Delta\Delta Cq}$ method was used to process all data.

Cell line, cell culture, and transfection

Two human NSCLC cell lines – NCI-H1993 [H1993] and NCI-H2170 [H2170] – and a normal human lung cell line – IMR-90 – were purchased form the American Type Culture Collection (ATCC, USA). All cells were cultured with ATCC-formulated RPMI-1640 medium (ATCC 30-2001) containing 10% fetal bovine serum (FBS, ATCC 30-2020) in an incubator (37°C, 5% CO_2). AWPPH cDNA was amplified by PCR reactions

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Table 1. Basic information of participants in the 4 groups.

	Control	NR (54)	LR (42)	DR (32)
Gender				
Male	16	28	20	15
Female	14	26	22	17
Age range (years)	23–66	25–65	22–68	26
Mean age (years)	45.1±6.2	44.4±5.6	43.8±5.7	44.9±6.1
Histological type				
Adeno-Ca	NA	30	24	17
Squamous-Ca	NA	20	16	10
Adenosquamous-Ca	NA	5	4	3
Nodal involvement				
N0	NA	20	16	10
N1	NA	21	17	14
N2	NA	11	8	5
NX	NA	3	2	1
Tumor stage				
Stage 1	NA	19	17	10
Stage 2	NA	18	13	7
Stage 3	NA	12	9	7
Stage 4	NA	7	5	4
Histological grade				
G1	NA	4	2	2
G2	NA	23	19	14
G3	NA	27	20	17

and was inserted into pIRSE2 vector (Clontech, Palo Alto, CA, USA) to make the AWPPH expression vector. Lipofectamine 2000 reagent (11668-019, Invitrogen, Carlsbad, USA) was first mixed with vector to allow the formation of reagent-vector complexes, followed by the incubation with cells at 37°C to achieve transfection. Cells without tranfection were used as the control group (C). Cells transfected with empty vector were used as the negative control. Overexpression rate above 200% was confirmed by qRT-PCR before subsequent experiments.

In vitro migration and invasion assay

A cell suspension was made at a cell density of 4×10^4 cells/ml using RPMI-1640 medium. The upper chamber was filled with 0.1 ml cell suspension (1% FBS), while the lower chamber was filled with RPMI-1640 medium (Thermo Fisher Scientific, USA) containing 20% FBS (Sigma-Aldrich, USA). Cells were cultured for 24 h. Membranes were then collected, cleaned, and stained with 0.5% crystal violet (Sigma-Aldrich, USA) at 25°C for 15 min. Matrigel (356234, Millipore, USA) was used to coat the upper chamber before the invasion assay. Migrating and invading cells were counted under an optical microscope. Cell migration and invasion were normalzied to the control group.

Western blot analysis

RIPA solution (Thermo-Fisher Scientific) was used to extract total protein. All operations were performed in strict accordance with the instructions of the kit. BSA assay was performed to measure protein concentration. Protein samples were mixed with loading buffer with a ratio of 1: 5 and were denatured at 85°C for 1 h. SDS-PAGE gel electrophoresis was performed with 35 μg protein in each well. After gel transfer to PVDF membranes, membranes were blocked with 5% skimmed milk at 25°C for 1 h, followed by incubation with rabbit anti-human primary antibodies of TGF-β1 (ab9758, 1: 1500; Abcam) and

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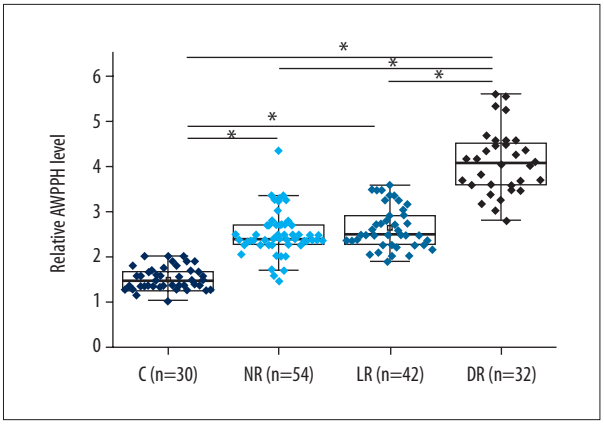

Figure 1. Comparison of blood levels of AWPPH among the 4 groups of participants on the day of admission. Blood AWPPH levels were highest in the DR group, followed by LR and NR groups, and the lowest blood AWPPH levels were observed in the control group. * p<0.05.

GAPDH (rabbit anti-human, ab9485, 1: 1400, Abcam) at 4°C overnight. Membranes were then incubated with IgG-HRP secondary antibody (1: 1000, MBS435036, MyBioSource) at 25°C for 2 h. ECL (Sigma-Aldrich, USA) was then used to develop signals, and the grey band of TGF-β1 was normalized to that of GAPDH using Image J software to represent relative TGF-β1 expression level.

Statistical analysis

GraphPad Prism 6 software was used to perform all statistical analyses. All data were recorded as mean ± standard deviation. Data were compared by one-way analysis of variance (ANOVA) followed by the LSD test (among multiple groups) and *t* test (between 2 groups). Correlation analyses were performed by Pearson correlation analyses. p<0.05 was considered to be statistically significant.

Results

Blood lncRNA AWPPH levels were higher in DR group patients on the day of discharge

QRT-PCR was performed to detect the expression of AWPPH in blood of all 3 patient groups and the control group. As shown in Figure 1, the highest blood AWPPH levels were observed in DR group patients (p<0.05). Compared with the control group, significantly upregulated expression of AWPPH was also found in the NR and LR groups (p<0.05).

Blood lncRNA AWPPH levels increased significantly in the DR group but not in other groups of patients

During follow-up, blood was extracted from 74 patients on the day of the diagnosis of recurrence (patients in LR and DR groups) and 54 patients at the end of follow-up (NR group). Compared with the levels on the day of discharge, AWPPH levels were increased significantly in the DR group (Figure 2A, p<0.05) but not in the LR (Figure 2B, p>0.05) and NR (Figure 2C, p>0.05) groups.

Blood lncRNA AWPPH levels were positively correlated with blood TGF-β1 mRNA in the DR group but not in other groups during follow-up

TGF-β signaling plays pivotal roles in cancer metastasis [6]. Pearson correlation was used to analyze the correlations between blood lncRNA AWPPH and TGF-β1 mRNA levels. As shown in Figure 1, a significant positive correlation was found between blood levels of AWPPH and TGF-β1 mRNA in the DR group. In contrast, no significant correlations were found between blood levels of AWPPH and TGF-β1 in the LR (Figure 3B) and NR (Figure 3C) groups.

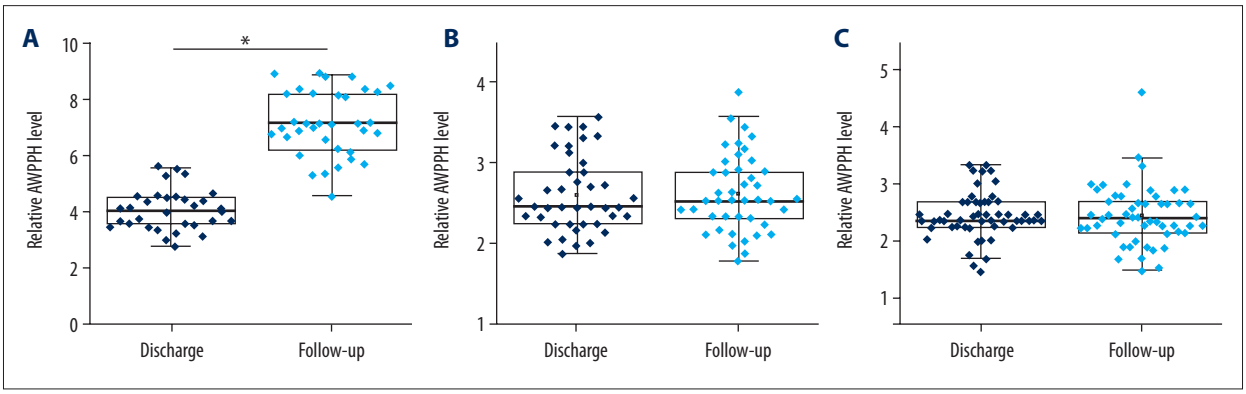

Figure 2. Blood lncRNA AWPPH levels increased significantly in the DR group but not in other groups of patients. The comparison of blood levels of lncRNA AWPPH on the day of discharge and during follow-up in DR (**A**), LR (**B**), and NR (**C**) groups. * p<0.05.

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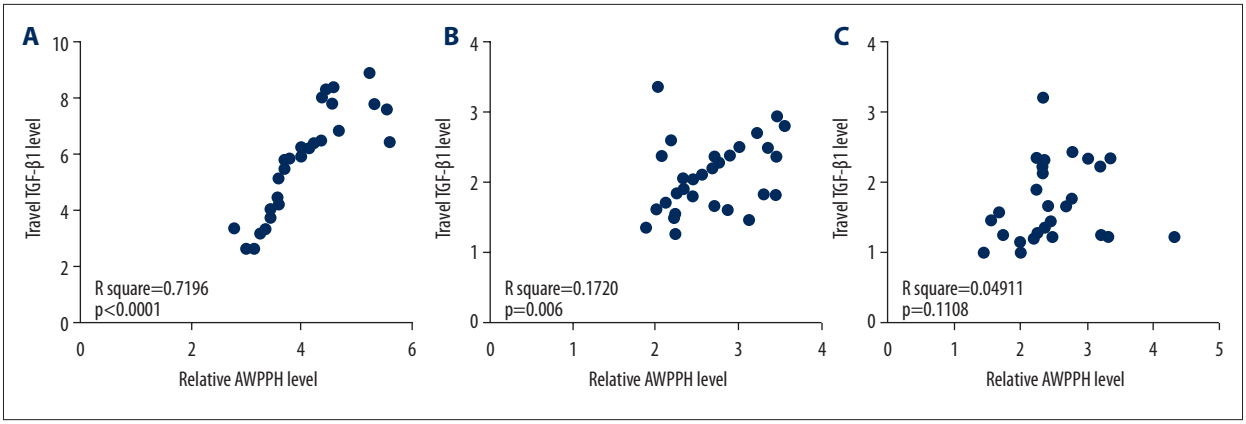

Figure 3. Blood lncRNA AWPPH levels were positively correlated with blood TGF-β1 mRNA in the DR group but not in other groups during follow-up. Pearson correlation analysis of the correlations between blood lncRNA AWPPH and TGF-β1 mRNA levels in DR (**A**), LR (**B**), and NR (**C**) groups.

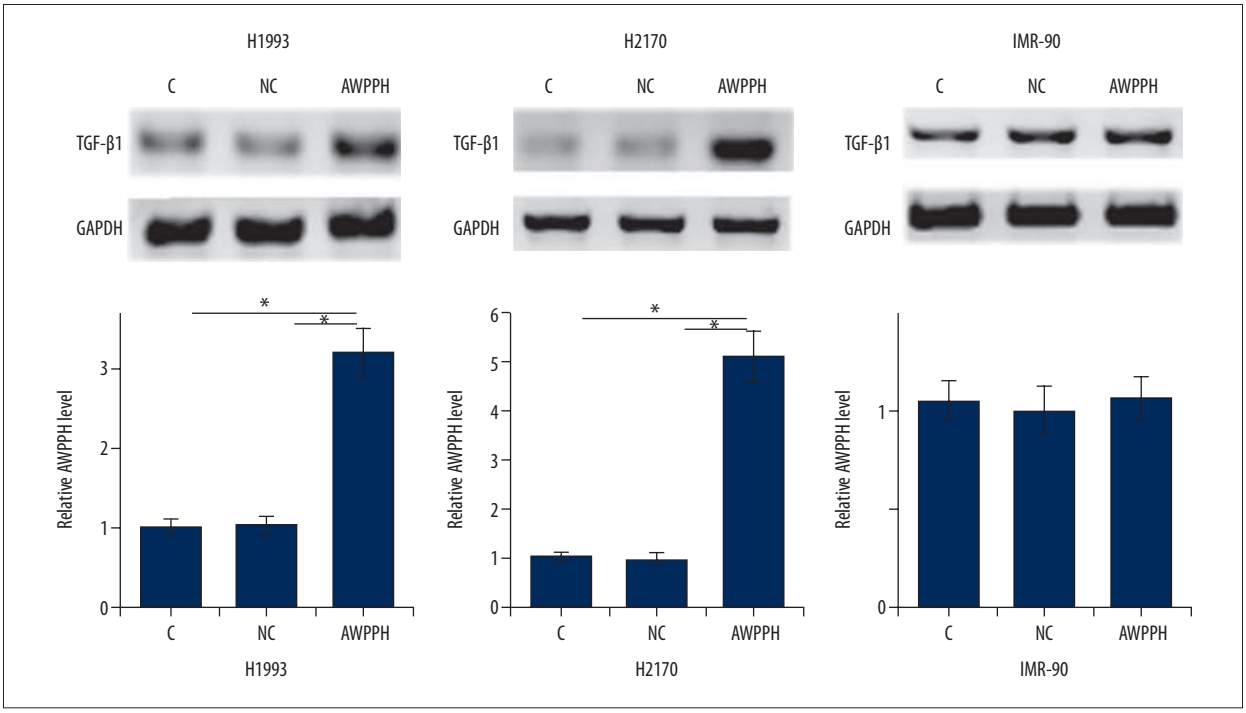

Figure 4. AWPPH overexpression upregulated TGF-β1 protein expression. AWPPH overexpression significantly upregulated AWPPH expression in NSCLC cells but not in normal lung cells. TGF-β1 treatment had no significant effects on AWPPH expression. * p<0.05.

AWPPH overexpression upregulated TGF-β1 protein expression

To further investigate the interactions between AWPPH and TGF-β1, AWPPH expression vectors were transfected into NSCLC cells, and the effects of AWPPH overexpression on TGF-β1 expression were explored by Western blot analysis. As shown in Figure 4, AWPPH overexpression significantly upregulated AWPPH expression in cells of 2 human NSCLC cell lines – H1993 and H2170 (p<0.05) – but not in cells of the normal lung cell line – IMR-90 (p>0.05). In contrast, treatment with exogenous TGF-β1 (Sigma-Aldrich) at doses of 10, 20, 40, and 80 ng/ml showed no significant effects on AWPPH expression in cells of the 3 cell lines.

AWPPH overexpression promoted NSCLC cell migration and invasion

In vitro cell migration and invasion after AWPPH overexpression were explored by Transwell migration and invasion assays.

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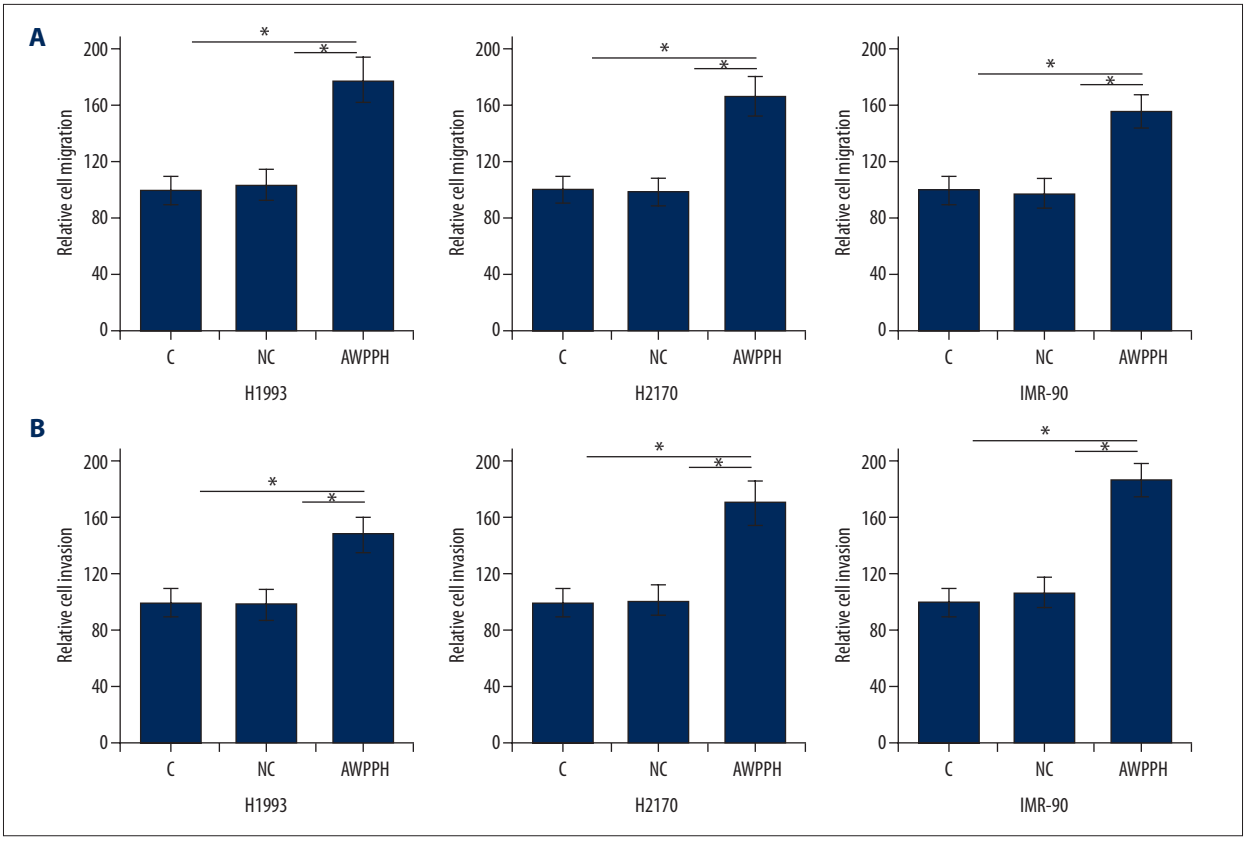

Figure 5. (**A, B**) AWPPH overexpression promoted NSCLC cell migration and invasion. Effects of AWPPH overexpression on cell migration and invasion of different cell lines. AWPPH overexpression promoted migration and invasion of cells of 2 human NSCLC cell lines – H1993 and H2170 – but not cells of the normal lung cell line – IMR-90.

Compared with control cells (C) without tranfection and negative control cells transfected with empty vector, NSCLC cells transfected with AWPPH expression vectors showed significantly increased migration (Figure 5A, $p<0.05$) and invasion (Figure 5B, $p<0.05$) abilities. However, AWPPH overexpression showed no significant effects on migration (Figure 5A, $p>0.05$) or invasion (Figure 5B, $p>0.05$) of cells of the normal lung cell line IMR-90.

Discussion

AWPPH is a recently identified oncogenic lncRNA with upregulated expression in hepatocellular carcinoma [11] and bladder cancer [12]. The key finding of our study is that the upregulated AWPPH expression is closely correlated with postoperative distant recurrence of NSCLC. The mechanism of the action of AWPPH is likely achieved through the upregulation of TGF-β1.

More and more studies have proved than lncRNAs are key players in nearly all aspects of cancer development and progression [13]. Postoperative recurrence is a major cause of cancer-related deaths, but there have been few studies on cancer recurrence after surgical resection. A previous study showed that the overexpression of lncRNA MALAT-1 is closely correlated with tumor recurrence in hepatocellular carcinoma patients after liver transplantation [14], but the mechanism of the function of MALAT-1 in this process is unknown. In another study, a large set of differentially-expressed lncRNAs in recurrent gliomas and primary gliomas were identified [15]. In a study on NSCLC, Ono et al. reported that upregulated expression of lncRNA HOTAIR is closely correlated with tumor clinical relapse [16]. In the present study, we focused on the blood level of lncRNA on the day of discharge and by the time of the diagnosis of recurrence. Significantly higher expression of AWPPH was observed in patients with distant recurrence than in patients with local recurrence and in patients without recurrence at both time points. Those data suggest that AWPPH is specifically involved in distant recurrence of resected NSCLC. Our data also suggest that the pathological mechanisms of local and distant recurrence are different.

TGF-β signaling plays pivotal roles in the development and progression of different types of cancers [6–8]. In a recent study, Calon et al. reported that increase in overall TGF-β expression leads to significantly increased cancer recurrence rate in

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patients with colorectal cancer [17]. In another study, mutations in genes involved in TGF-β signaling pathway were observed in 12% of recurrent breast tumors [18]. Although we do not show the data, we found that blood levels of TGF-β1 were significantly higher in patients with distant recurrence than in patients with local recurrence and in patients without recurrence (Figure 3), indicating the involvement of TGF-β1 in distant recurrence of NSCLC. TGF-β family signaling achieves its biological roles through both Smad-dependent and Smad-independent pathways [19]. In the development of bladder cancer, AWPPH inhibits SMAD4 via EZH2 to promote cancer development [12], indicating the potential crosstalk between AWPPH and TGF-β signaling. Our study shows that AWPPH is likely an upstream activator of TGF-β1. This conclusion is made based on the following 2 factors: 1) AWPPH promoted TGF-β1 expression, and 2) TGF-β1 did not significantly change AWPPH expression. It is also worth noting that AWPPH overexpression showed significant effects on the biological behaviors of normal lung cells. Therefore, AWPPH may serve as a therapeutic target for the prevention of postoperative distant recurrence in resected NSCLC.

We found that recurrence occurred in 74 of 128 (57.87%) patients. However, many patients (about 80) with good conditions after treatment failed to complete follow-up. About 90% of patients were at stage 1 or stage 2. Therefore, the real recurrence rate is lower than 57.87%.

Conclusions

AWPPH overexpression is involved in the postoperative distant recurrence of resected NSCLC. This action of AWPPH is likely achieved through upregulation of TGF-β1 and the promoted cancer cell migration and invasion.

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