European Review for Medical and Pharmacological Sciences 2020; 24: 10445-10451

High expression of WISP1 promotes metastasis and predicts poor prognosis in hepatocellular carcinoma

Q.-Y. WANG[1], Y.-J. FENG[1], R. JI[2]

[1]Department of GI Medicine, Rizhao People's Hospital, Rizhao, China
[2]Department of GI Medicine, Qilu Hospital, Jinan, China

Qinyi Wang and Yujia Feng contributed equally to this work

Abstract. – OBJECTIVE: The aim of this study was to explore the expression level of Wnt1-inducible signaling pathway protein 1 (WISP1) and its clinical significance in hepatocellular carcinoma (HCC).

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to detect the expression level of WISP1 in HCC tissues and cells. Kaplan-Meier curves and Cox proportional hazard regression model were chosen for single and multiple factor analysis of survival analysis, respectively. Furthermore, wound healing assay and transwell assay were used to verify the effect of WISP1 on HCC cell metastasis *in vitro*.

RESULTS: The expression level of WISP1 in HCC tissues was significantly higher than that in para-cancer tissues ($p<0.05$). WISP1 expression was positively correlated with lymph node metastasis and clinical stage of HCC. Kaplan-Meier curve showed that HCC patients with higher WISP1 expression exhibited significantly worse progression free survival (PFS) time and overall survival (OS) time. Both univariate and multivariate analysis indicated that high expression of WISP1 was an independent predictor of poor prognosis in HCC. In addition, WISP1 significantly promoted the invasion and migration of HCC cells *in vitro*.

CONCLUSIONS: WISP1 might contribute to the development of HCC, serving as a clinical biomarker and therapeutic target for HCC patients.

Key Words:
Hepatocellular carcinoma (HCC), Wnt1-inducible signaling pathway protein 1 (WISP1), Overall survival (OS), Progression free survival (PFS).

Introduction

Liver cancer is a common malignant tumor of the digestive system. As the most common type, hepatocellular carcinoma (HCC) accounts for over 90% of all types of liver cancer[1]. The incidence rate of HCC ranks 6th among common malignant tumors worldwide. Meanwhile, its mortality rate ranks 2nd, exhibiting an uptrend[2]. In the context of diversified treatment methods for HCC, the 5-year survival rate of patients has not been significantly improved, with the overall survival (OS) rate lower than 5%[3]. On one hand, the reason is that HCC possesses strong abilities to invade adjacent organs and metastasize to distant organs with quite low sensitive to current conventional radiotherapy, chemotherapy, targeted drug therapy, and other therapies[4]. On the other hand, there is still a lack of clinical target that can effectively improve the early diagnosis and curative effect of patients. Currently, comprehensive treatment based on surgical resection has greatly prolonged the 1-2-year survival rate of HCC patients. However, the 3-5-year OS rate is still far from satisfactory[5].

Wnt1-inducible signaling pathway protein 1 (WISP1) is a secretory extracellular matrix-related protein belongs to the Cyr61-CTGF-Nov (CCN) growth factor family[6]. WISP1 plays important roles in many biological processes, such as cell adhesion, chondrogenesis, angiogenesis, and tumorigenesis[7-9]. It acts as a crucial target gene downstream of the Wnt/β-catenin pathway, which is regulated by different signaling pathways. It is sensitive to different extracellular biochemical signals and can facilitate β-catenin-mediated tumorigenesis[10]. Furthermore, WISP1 signal inhibits p53-dependent apoptosis not through the Fas ligand activation pathway, activates the Akt anti-apoptotic signaling pathway, suppresses mitochondria from releasing cytochrome C, and upregulates anti-apoptotic protein B-cell lym-

Corresponding Author: Qinyi Wang, MM; e-mail: quzhongrenbusan07@163.com

phoma extra-large (Bcl-xL) to prevent cells from apoptosis induced by DNA damage[11]. Therefore, WISP1 is closely related to the occurrence, development, and prognosis of various tumors.

In the present study, we aimed to explore the expression level of WISP1 and its clinical significance in HCC. Our findings hoped to contribute to clarify the biological mechanism of HCC, as well as providing valuable targets for its diagnosis and treatment.

Patients and Methods

Tissue Specimens Collected

A total of 80 pairs of paraffin-embedded HCC tissues and adjacent normal tissues were collected from patients undergoing surgical resection from April 2016 to December 2018 in Rizhao People's Hospital. Follow-up referred to the interval between the operation date and the death date or the last follow-up date. All patients were followed up every 3 months until the 5th year, and these patients had complete follow-up data before they died, or the data updated to the latest follow-up. TNM staging was carried out for HCC according to the standard of the American Joint Committee on Cancer. This study was approved by the Ethics Committee of Rizhao People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human HCC SMMC-7721 cells were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT, USA), 100 Ul/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Cell Transfection

Transfection was performed when the cells were in the logarithmic phase. Briefly, the cells were inoculated into 6-well plate at a density of 1×10^6 cells/well (2 mL). Subsequently, the cells were transfected with si-WISP1 or si-NC (negative control) according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). The total RNA was extracted in the cells after 48 h of transfection for later use.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNAs in HCC tissues and cells were extracted in accordance with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reversely transcribed into cDNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative expression level of WISP1 was finally calculated. The primer sequences used in this study were as follows: WISP1, F: 5'-GCAGGTACCGGCTCGACTGC-3', R: 5'-GACGGTAGGGACTCCCAGGGA-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Wound Healing Assay

Transfected SMMC-7721 cells for 48 h were cultured in 6-well plates overnight until the fusion degree reached 90%. The original culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) solution. After that, a 20 μL spear head was utilized to make cell scratches, and the residual cells were washed with PBS solution. Next, the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 5% serum for 48 h. The healing of cell scratches was finally observed under an inverted microscope.

Transwell Assay

DMEM culture solution was first diluted with Matrigel at a ratio of 1:8, and the upper surface of the transwell chamber membrane (100 μL/well) was coated. Transfected SMMC-7721 cells for 72 h were selected and re-suspended in serum-free culture medium until the density reached 5×10^5 cells/mL. The upper chamber was added with 250 μL of cell suspension. Meanwhile, the lower chamber was added with complete culture medium containing 10% fetal bovine serum. Then, the cells were incubated in an incubator with 5% CO_2 at 37°C for 12 h. Next, non-invasive cells on the membrane and Matrigel were wiped out with cotton swabs, followed by rinsing twice with PBS solution. The cells were fixed with 4% paraformaldehyde and stained with crystal violet dye. Finally, invasive cells were observed under a microscope, and the number of invasive cells was counted. 10 fields of view were randomly selected for each sample.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was selected for all statistical analysis. Measurement data were expressed as mean ± standard deviation ($\chi \pm s$) or median. Paired *t*-test was used to compare the expression level of WISP1 in primary HCC tissues and adjacent normal tissues. Group χ^2-test was adopted to analyze the associations of WISP1 expression in HCC tissues with clinicopathological features of patients. Overall survival (OS) and progression free survival (PFS) of HCC patients was evaluated *via* Kaplan-Meier survival analysis, and the intergroup differences were analyzed by Log-rank test. Cox proportional hazard regression models were respectively chosen for single factor analysis and multiple factor analysis of survival analysis. $p<0.05$ was considered statistically significant.

Results

Effect of WISP1 on the Prognosis of Patients with HCC

The relationship between WISP1 expression and PFS and OS time in 80 HCC patients was analyzed by Kaplan-Meier method. HCC patients with high expression of WIPS1 exhibited significantly worse prognosis. FPS and OS of HCC patients with high expression of WIPS1 was about 13% (FPS) and 18% (OS) shorter than those with low expression, respectively. These findings indicated that high expression of WISP1 predicted poor prognosis of HCC patients (Figure 1).

Expression of WISP1 in HCC Tissues

The expression of WISP1 in 80 pairs of HCC tissues and adjacent normal tissues was detected. The results were similar to those reported in other literatures[12,13]. Compared with adjacent normal tissues, the expression of WISP1 significantly increased in HCC tissues (about 2 times higher). The expression of WISP1 in normal tissues and HCC tissues was 0.7615 ± 0.118 and 1.828 ± 0.2337, respectively (Figure 2A).

Based on the median expression of WISP1, HCC patients were divided into two groups, including: high expression group (WISP1 expression level > 1.828, n=40) and low expression group (WISP1 expression level < 1.828, n=40). Subsequently, clinical features were selected to analyze whether they were associated with the expression level of WISP1 in HCC tissues. Our results demonstrated that WISP1 expression was positively correlated with lymph node metastasis and clinical stage of HCC (Table I).

Univariate Analysis and Multivariate Analysis of WISP1 Expression and HCC Clinicopathological Data

Univariate Cox proportional hazards regression model analysis revealed that tumor size, tumor number, lymph node metastasis, TNM stage, and WISP1 expression level were independent risk factors for poor prognosis of HCC patients. All the above indicators were validated to be associated with poor prognosis of patients with HCC by multivariate Cox proportional hazard model analysis. These findings suggested that WISP1 promoted HCC development, which could be used as a new index to predict the prognosis of HCC (Table II).

Effect of WISP1 on the Migration and Invasion of HCC Cells

Migration and invasion are the most basic characteristics of distant metastasis of cancer cells[14]. In this study, we established a WIPS1 low expression

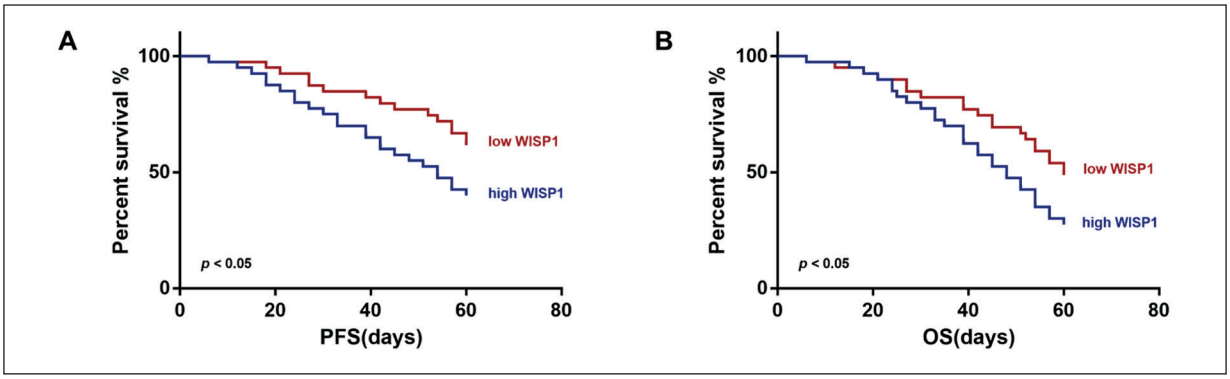

Figure 1. The relationship of WIPS1 expression with progression-free survival (PFS) (**A**) and overall survival (OS) (**B**) of HCC patients.

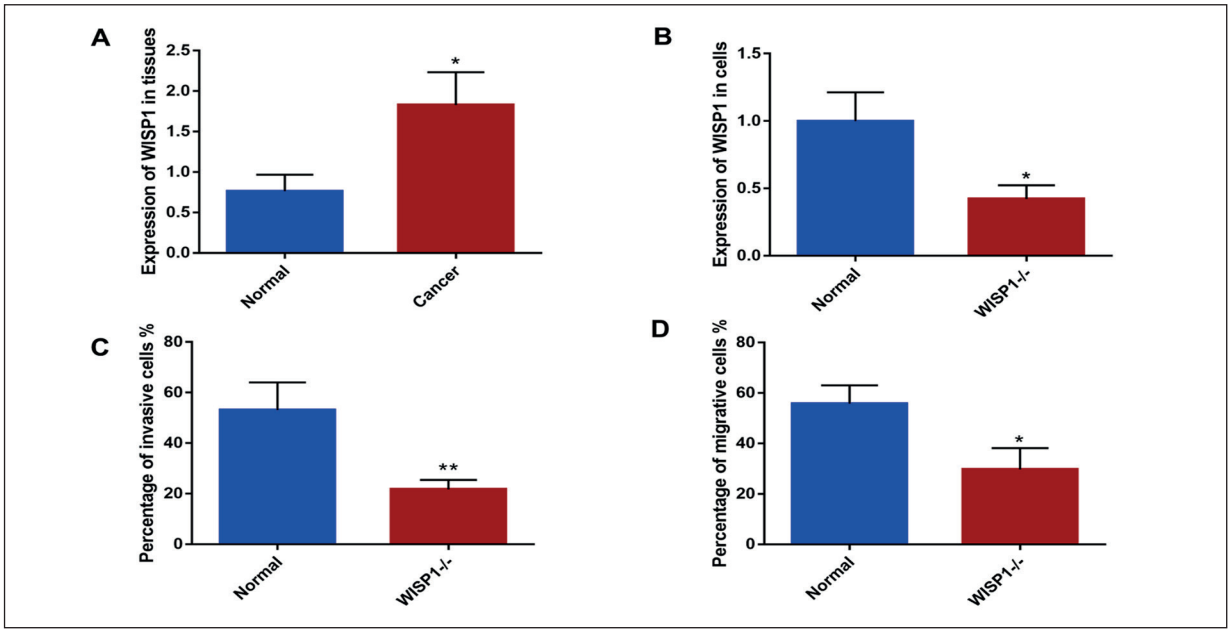

Figure 2. A, The expression level of WIPS1 was measured in HCC tissues and adjacent normal tissues by qRT-PCR. **B,** Transfection efficiency verified by qRT-PCR. **C,** and **D,** WIPS1 promoted the invasion and migration of HCC cells. (*p<0.05, **p<0.01).

model by si-WIPS1 transfection *in vitro* (Figure 2B). Transwell assay found that the migration and invasion of HCC cells with low expression of WIPS1 were significantly impaired. All these findings suggested that WIPS1 promoted the metastasis ability of HCC cells (Figure 2C-2D).

Table I. WISP1 expression and clinical features of patients with HCC.

Features	No.	WISP1 expression level		p
		High	Low	
No. gender	80	40	40	0.1095
Male	48	20	28	
Female	32	20	12	
Age (years)				0.2611
< 60	36	21	15	
≥ 60	44	19	25	
AFP				0.8027
< 20	22	10	12	
≥ 20	58	30	28	
HBsAg				0.2247
Negative	13	4	9	
Positive	67	36	31	
Tumor size (cm)				0.0619
< 5	29	19	10	
≥ 5	51	21	30	
Tumor number				0.1471
Solitary	55	24	31	
Multiple	25	16	9	
Lymph node metastasis				
0.0001***				
Absence	42	11	31	
Presence	38	29	9	
Clinical stage				0.0060**
I + II	47	30	17	
III + IV	33	10	23	

Table II. Univariate and multivariate analyses of postoperative prognosis in patients with HCC.

Features	Univariate analysis		Multivariate analysis	
	Hazard ratio/CI (95%)	p	Hazard ratio/CI (95%)	p
Gender	0.883/0.654-1.138	0.768		
Ages	1.076/0.813-1.199	0.913		
HBsAg	1.089/0.776-1.340	0.865		
AFP	1.103/0.813-1.199	0.913		
Tumor size	1.627/0.850-3.512	0.041*	1.442/0.704-3.433	0.049*
Tumor number	1.655/0.874-2.996	0.035*	1.507/0.722-2.842	0.042*
Lymph node metastasis	2.220/1.038-4.142	0.014*	2.106/1.011-3.507	0.026*
TNM stage	3.737/2.576-5.371	0.005**	3.135/2.326-4.822	0.011*
WISP1 expression level	1.694/1.070-3.233	0.033*	1.484/0.898-3.004	0.044*

Discussion

The occurrence and development of cancer is a multi-stage process, including malignant proliferation, adhesion, invasion, metastasis, and angiogenesis. Multiple genes have been found involved in the development and progression of malignancies. Invasion and migration of tumor cells is a multi-factor and multi-step complex process. Hanahan et al[15] and Hainaut et al[16] have indicated that metastasis is the core problem in cancer treatment. Tumor metastasis occurs after a series of complex cellular biological events, which are collectively referred to invasion and metastasis cascade reactions. In other words, epithelial cells in primary tumors locally invade through the surrounding extracellular matrix (ECM) to diffuse from primary tumors and enter the circulatory system. After successful metastasis, some circulating tumor cells (CTCs) seep out of the vasculature and begin distant metastasis and colonization. Tumor cells need to adapt to the new microenvironment and transform from migration mode to proliferation mode, enabling tumors detected microscopically and clinically. Cell metastasis is a vital dynamic process in every multi-cellular organism, especially in organisms lacking cell walls. Meanwhile, it is also the center of morphogenesis. Cell metastasis has been confirmed essential for wound healing and the maintenance of tissue homeostasis, renewal, and integrity. The above processes are strictly controlled *in vivo*. Once they are deviated from the normal orbit, excessive cell transfer may lead to severe pathological conditions, such as tissue disintegration or fibrosis. An evident example of pathological results caused by cell transfer disorder is that cancer cells diffuse from primary tumors and form secondary tumors and metastatic foci in distant organs and tissues. The ability of cancer cells to penetrate extracellular matrix and invade surrounding tissues require differentiated cells to transform into cells with migration and invasion phenotypes. This transformation is always accompanied by changes in cell shapes, which are the origin of most solid tumors.

The expression of WISP1 has been found to play a promoting role in tumor growth. Compared with healthy organs, WISP1 is highly expressed in various tumors. WISP1 was first found in a cancer cell line model and was then detected in a variety of cancer tissues *in vivo*. Chuang et al[17] have found that WISP1 upregulates the expression of ICAM-1 through integrin αvβ3 receptor, as well as ASK1, JNK/p38, and AP-1 signal transduction pathways, thus promoting the development of oral squamous cell carcinoma. In addition, WISP1 induces the expression of VEGF-A and trans-activates the EGFR/ERK/HIF1-α signaling pathway. This may in turn triggers the accumulation of endothelial cells and the formation of new blood vessels in tumor microenvironment[18]. Yang et al[19] detected WISP1 expression in 194 pancreatic cancer tissues using immunohistochemical techniques and have found that WISP1 is highly expressed in pancreatic cancer. High expression of WISP1 is closely related to clinical T stage and liver metastasis, indicating that WISP1 is expected to be a prognostic marker for pancreatic cancer patients. Additionally, the expression of WISP1 in colon tumor-associated fibroblasts (CAFs) is prominently raised. Upregulation of its protein expression level is closely associated with the pathological stage and prognosis of colon cancer patients[20]. In breast cancer patients, overexpressed WISP1 cannot only contribute to the proliferation, invasion, and metastasis of breast cancer cells and epithelial-mesenchy-

mal transition, but also suppresses the expression of tumor suppressor gene NDRG1[21]. Jia et al[22] have demonstrated that elevated expression of WISP-1 boosts the proliferation, invasion, and migration of gastric cancer BGC-823 and AGS cells. As a WISP1-related gene, CyclinD1 is capable of accelerating the proliferation of gastric cancer cells. Moreover, WISP1 stimulates tumor invasion and migration through epithelial-mesenchymal transition of gastric cancer cells.

In this study, we found that the expression level of WISP1 in HCC tissues was significantly upregulated. After analyzing the correlation between the expression of WISP1 with clinical pathological features of HCC patients, our finding showed that high expression of WISP1 was positively associated with lymph node metastasis and clinical stage. Cox proportional hazard regression model demonstrated that WISP1 served as an independent risk factor for the prognosis of HCC patients, which was the same as tumor size, tumor number, lymph node metastasis, and TNM stage. *In vitro*, we verified the promotion effect of WISP1 on the migration and invasion of HCC cells.

Conclusions

The results of this study showed that WISP1 significantly promoted the progression of HCC. High expression of WISP1 was correlated with the metastatic ability of HCC cells. However, the specific role and molecular mechanism of WISP1 in HCC remained to be further explored.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65: 5-29.
2) Jin XL, Lian JR, Guan YH. Overexpression of long non-coding RNA MINCR contributes to progressive clinicopathological features and poor prognosis of human hepatocellular carcinoma. Eur Rev Med Pharmacol Sci 2018; 22: 8197-8202.
3) Liu L, Zheng YH, Han L, Qin SK. Efficacy and safety of the oxaliplatin-based chemotherapy in the treatment of advanced primary hepatocellular carcinoma: a meta-analysis of prospective studies. Medicine (Baltimore) 2016; 95: e4993.
4) Bruix J, Sherman M; American Association for the Study of Liver Diseases. Management of hepatocellular carcinoma: an update. Hepatology 2011; 53: 1020-1022.
5) Lencioni R, Chen XP, Dagher L, Venook AP. Treatment of intermediate/advanced hepatocellular carcinoma in the clinic: how can outcomes be improved? Oncologist 2010; 15 Suppl 4: 42-52.
6) Berschneider B, Konigshoff M. WNT1 inducible signaling pathway protein 1 (WISP1): a novel mediator linking development and disease. Int J Biochem Cell Biol 2011; 43: 306-309.
7) Venkatachalam K, Venkatesan B, Valente AJ, Melby PC, Nandish S, Reusch JE, Clark RA, Chandrasekar B. WISP1, a pro-mitogenic, pro-survival factor, mediates tumor necrosis factor-alpha (TNF-alpha)-stimulated cardiac fibroblast proliferation but inhibits TNF-alpha-induced cardiomyocyte death. J Biol Chem 2009; 284: 14414-14427.
8) Shanmugam P, Valente AJ, Prabhu SD, Venkatesan B, Yoshida T, Delafontaine P, Chandrasekar B. Angiotensin-II type 1 receptor and NOX2 mediate TCF/LEF and CREB dependent WISP1 induction and cardiomyocyte hypertrophy. J Mol Cell Cardiol 2011; 50: 928-938.
9) Tanaka S, Sugimachi K, Saeki H, Kinoshita J, Ohga T, Shimada M, Maehara Y, Sugimachi K. A novel variant of WISP1 lacking a Von Willebrand type C module overexpressed in scirrhous gastric carcinoma. Oncogene 2001; 20: 5525-5532.
10) Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. Genes Dev 2000; 14: 585-595.
11) Wang S, Chong ZZ, Shang YC, Maiese K. Wnt1 inducible signaling pathway protein 1 (WISP1) blocks neurodegeneration through phosphoinositide 3 kinase/Akt1 and apoptotic mitochondrial signaling involving Bad, Bax, Bim, and Bcl-xL. Curr Neurovasc Res 2012; 9: 20-31.
12) Wu J, Long Z, Cai H, Du C, Liu X, Yu S, Wang Y. High expression of WISP1 in colon cancer is associated with apoptosis, invasion and poor prognosis. Oncotarget 2016; 7: 49834-49847.
13) Ono M, Inkson CA, Sonn R, Kilts TM, de Castro LF, Maeda A, Fisher LW, Robey PG, Berendsen AD, Li L, McCartney-Francis N, Brown AC, Crawford NP, Molinolo A, Jain A, Fedarko NS, Young MF. WISP1/CCN4: a potential target for inhibiting prostate cancer growth and spread to bone. PLoS One 2013; 8: e71709.
14) Wu W, Liu X, Wei L, Li T, Zang Y, Qian Y, Bai T, Li J, Xie M, Zhu Y, Wang Q, Wang L. Tp53 mutation inhibits ubiquitination and degradation of WISP1 via down-regulation of Siah1 in pancreatic carcinogenesis. Front Pharmacol 2018; 9: 857.
15) Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-674.
16) Hainaut P, Plymoth A. Targeting the hallmarks of cancer: towards a rational approach to next-gen-

eration cancer therapy. Curr Opin Oncol 2013; 25: 50-51.

17) Chuang JY, Chang AC, Chiang IP, Tsai MH, Tang CH. Apoptosis signal-regulating kinase 1 is involved in WISP-1-promoted cell motility in human oral squamous cell carcinoma cells. PLoS One 2013; 8: e78022.

18) Chuang JY, Chen PC, Tsao CW, Chang AC, Lein MY, Lin CC, Wang SW, Lin CW, Tang CH. WISP-1 a novel angiogenic regulator of the CCN family promotes oral squamous cell carcinoma angiogenesis through VEGF-A expression. Oncotarget 2015; 6: 4239-4252.

19) Yang JY, Yang MW, Huo YM, Liu W, Liu DJ, Li J, Zhang JF, Hua R, Sun YW. High expression of WISP-1 correlates with poor prognosis in pancreatic ductal adenocarcinoma. Am J Transl Res 2015; 7: 1621-1628.

20) Rupp C, Scherzer M, Rudisch A, Unger C, Haslinger C, Schweifer N, Artaker M, Nivarthi H, Morriggl R, Hengstschlager M, Kerjaschki D, Sommergruber W, Dolznig H, Garin-Chesa P. IGFBP7, a novel tumor stroma marker, with growth-promoting effects in colon cancer through a paracrine tumor-stroma interaction. Oncogene 2015; 34: 815-825.

21) Chiang KC, Yeh CN, Chung LC, Feng TH, Sun CC, Chen MF, Jan YY, Yeh TS, Chen SC, Juang HH. WNT-1 inducible signaling pathway protein-1 enhances growth and tumorigenesis in human breast cancer. Sci Rep 2015; 5: 8686.

22) Jia S, Qu T, Feng M, Ji K, Li Z, Jiang W, Ji J. Association of Wnt1-inducible signaling pathway protein-1 with the proliferation, migration and invasion in gastric cancer cells. Tumour Biol 2017; 39: 1393389909.