

WISP1 and miR-29c-3p are novel prognostic biomarkers and therapeutic targets for bladder cancer

Lihua Zhang (✉ lihuazhang@xmu.edu.cn)

Xiamen University Medical College

Zhuo Wang

Wuxi People's Hospital

Hongyi Zhou

Wuxi People's Hospital

Ming Zhu

Wuxi People's Hospital

Leilei Zhu

Wuxi People's Hospital

Zhuoqun Xu

Wuxi People's Hospital

Jianfeng Shao

Wuxi People's Hospital

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Abstract

Background: Aberrant expression of WISP1 is associated with carcinogenesis; however, the expression and prognostic values of WISP1 in bladder cancer (BC) remain elusive. Therefore, the present study aimed to investigate the WISP1 expression in BC and explore the possible mechanisms and clinical value of WISP1 in BC.

Methods: The *in silico* analysis based on oncomine and Kaplan-Meier Plotter databases were to reveal WISP1 expression and prognosis in BC. Besides, qRT-PCR, immunohistochemistry (IHC) and western blot assays were used to detect WISP1 expression both in BC tissues and BC cell lines. TargetScan database was used to predict miRNAs that putatively regulate the expression of WISP1. COX regression and the Kaplan-Meier method were applied to evaluate the prognostic value of WISP1 and miRNAs in BC.

Results: WISP1 was up-regulated in BC and associated with poor survival in patients with BC through *in silico* analysis. Besides, WISP1 expression was identified to be up-regulated both in BC tissues and cell lines. We identified 13 miRNAs that putatively regulate the expression of WISP1. Of these miRNAs, only miR-29c-3p was found to be significantly negatively correlated with WISP1 in BC tissues. The correlation of *in situ* expressions of WISP1 and miR-29c-3p by immunohistochemistry (IHC) and clinical characteristics revealed that WISP1 was significantly associated with tumor size and hsa-miR-23b-3p expression, and miR-29c-3p was associated with tumor size, M stage, and WISP1 expression. Multivariate Cox regression analysis indicated that TNM stage and WISP1 expression were predictors of unfavorable prognosis, while hsa-miR-29c-3p was a predictor of favorable prognosis in patients with BC.

Conclusions: Collectively, the findings indicated that WISP1 and miR-29c-3p might serve as novel prognostic biomarkers and potential therapeutic targets for BC.

Introduction

Globally, bladder cancer(BC) ranks the tenth most frequently diagnosed urological malignancy, with approximately 550,000 new cases (nearly 425,000 in males and 125,000 in females) diagnosed in 2018 worldwide. It is among the leading causes of cancer-associated mortality, with approximately 200,000 deaths reported in 2018. Histologically, urothelial carcinoma (transitional cell) represents the predominant type of BC, accounting for 90 percent of all BC¹. Clinically, two main phenotypes, including the muscle-invasive and non-muscle-invasive, with different pathogenesis, molecular characteristics, and clinical outcomes, have been identified³. Recent advances in high-throughput sequencing technologies have facilitated the rapid molecular characterization and enhanced our understanding of the pathogenesis of BC, leading to the identification of actionable therapeutic targets for BC⁴. However, the molecular mechanisms underlying the occurrence and development of bladder carcinogenesis remain to be completely elucidated.

WNT1-inducible signaling pathway protein 1 (WISP1), a secreted matricellular protein, is a member of the connective tissue growth factor/CCN protein family, which is found in the extracellular matrix (ECM).

WISP1 is predominantly involved in various biological processes, including cell adhesion, proliferation, differentiation, survival, and carcinogenesis. The elevated expression of WISP1 has been detected in several cancers, including melanoma, glioblastoma, and hepatocellular carcinoma⁵⁻⁷. WISP1 expression has also been associated with tumor purity, an inflamed tumor microenvironment, advanced disease, EMT, and macrophage M2 polarization in multiple solid tumors⁸⁻¹¹. However, its expression and physiological function in BC remain elusive. MicroRNAs (miRNAs) are small 18–24 nucleotide long, single-stranded non-coding RNAs involved in post-transcriptional gene regulation by pairing to the mRNAs of its target protein-coding gene), thereby causing translational repression or mRNA degradation of the target gene¹². Recently, several miRNAs, such as miRNA-217 and miRNA-616^{13,14}, have been identified as prognostic biomarkers in BC^{15,16}. However, there is a paucity of studies on WISP1-related miRNAs in BC. Therefore, in the present study, we investigated the expression levels of WISP1 in BC using quantitative real-time PCR (qRT-PCR), Western blot assay, and bioinformatics analyses.

Furthermore, we used TargetScan to predict the potential WISP1-related miRNAs. Univariate and multivariate COX regression and the Kaplan-Meier method were applied to evaluate the prognostic value of WISP1 and miRNAs in BC.

Materials And Methods

Bioinformatics analysis

Oncomine (www.oncomine.org), an online microarray database, was used to analyze the mRNA levels of WISP1 in BC and normal bladder tissue samples^{17,18}. Kaplan-Meier Plotter (kmplot.com/analysis/) was used to analyze the prognostic value of WISP1 in BC¹⁹. TargetScan (7.2 version, <http://www.targetscan.org/>) was used to identify the potential miRNAs targeting WISP1²⁰.

BLAD Patient Samples

One hundred and thirty-two BC tissues and twenty paired BC tissues and adjacent normal tissues were obtained from patients who underwent surgical treatment for either diagnostic biopsy or surgical resection for BC at Wuxi People's Hospital Affiliated to Nanjing Medical University. The study protocols were approved by the Ethics Committee of the Institutional Review Board of Wuxi People's Hospital, Nanjing Medical University. Written informed consent was obtained from each patient. All protocols involving human patients were performed in accordance with the relevant guidelines and regulations of Wuxi People's Hospital, Nanjing Medical University. The tissue samples were immediately stored at -80°C until use. The histopathological examination was performed to confirm the diagnosis.

Cell Lines and Cell Culture

The human BC cell lines, including SV-HUC-1, UMUC3, RT4, T24, UC9, and 5637, were obtained by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in the Dulbecco's Modified Eagle's Medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS;

Invitrogen), 10% (100µg/mL) penicillin, and 100 U/mL streptomycin in a humidified atmosphere 5% CO₂ at 37°C.

Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was extracted from fresh frozen tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. cDNA was synthesized using a reverse transcription kit (PrimeScript RT-PCR kit; Takara, Japan) following the manufacturer's protocol. Total RNA was isolated from the cells using the PureLink™ RNA Mini Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Reverse transcription reactions were performed using iScript cDNA synthesis kit (Bio-Rad) for mRNA, or TaqMan miR Reverse Transcription kit (Applied Biosystems) for mature miR as described earlier. qRT-PCR was performed using SYBR Green Master Mix (Takara, Japan) on an ABI 7500 RealTime PCR System (Applied Biosystems, United States). β-actin and U6 were used as the reference genes. Primers used in the study were listed in Table 1. All reactions were performed in triplicate. The relative expression levels of miRNA and mRNA were evaluated using the 2^{-ΔΔCt} method.

Table 1 Primers of genes and miRNAs

Names	Forward	Reverse
WISP1	5'-GAAGCAGTCAGCCCTTATG-3'	5'-CTTGGGTGTAGTCCAGAAC-3'
β-actin	5'-CCTGTGGCATCCACGAAACT-3'	5'-GAAGCATTTGCG GTGGACGAT-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Western Blot assay

Total cell proteins were extracted from BC, and paracancerous tissues with RIPA lysis buffer (Pierce, Thermo Scientific, Cramlington, United Kingdom) supplemented with protease inhibitor cocktail. Protein concentrations were determined by BCA protein concentration reagent kit (Beyotime, China). Proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDA-PAGE) and transferred to PVDF membrane (Bio-Rad, USA). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature (RT). Subsequently, the membranes were incubated with the following primary antibodies: anti-WISP1 (Abcam, ab178547) and anti-β-actin (Abcam, ab8226) at 4°C for overnight. Then, membranes were washed and incubated with goat anti-rabbit IgG secondary antibody conjugated with horseradish peroxidase (1:1000). The target bands were visualized using an electrochemiluminescence (ECL) detection system. β-actin was used as an endogenous control.

Immunohistochemistry (IHC)

The immunohistochemical assay was performed on cancer tissues and adjacent normal tissues from BC patients. Tissue specimens were fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. The tissue section was cut into a thickness of 5 µm and then mounted on a glass slide. IHC staining was performed according to the manufacturer's protocol. In brief, slides were deparaffinized and then rehydrated in successively graded ethanol. Antigen retrieval was performed by heating the sections at

100°C for 30 minutes in the microwave. Subsequently, the slides were incubated with the primary antibody against WISP1 (diluted 1:200, ab178547, Abcam, USA) at 4°C overnight. The sections were washed 3 times in TBST for 5 minutes each and then incubated with the rabbit secondary antibody for 1 hour at room temperature. The sections were then stained with diaminobenzidine (DAB) and counterstained with hematoxylin. Three pathologists independently performed blinded analysis of the WISP1 immunostaining intensity under a light microscope. By analyzing the Based on the percentage of positively staining cells, the sections were grade as 0 = 5% or none of the cells were stained, 1 = 6-25% of the cells stained positive, 2 = 26-50% of the cells stained positive; 3 = 51-75% of the cells stained positive, and 4 = more than 76% of the cells stained positive. Similarly, the staining intensity was graded as 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. The two scores were multiplied, and the immune-reactive score (values from 0-12) for each case was determined²¹.

Statistical Analysis

All experiments were performed in triplicate. Data from three or more independent experiments were presented as the mean \pm standard deviation (SD). A paired Student's *t*-test was used to analyze the final score of cancer tissues and adjacent normal tissues. A Chi-square test was performed to assess the relationship between WISP1 and miRNAs expression and clinicopathological characteristics. The Kaplan-Meier method was used to calculate survival functions, and differences were compared using the log-rank test. Univariate and multivariate Cox proportional hazards analyses were used to identify the independent prognostic factors for overall survival in BC. Statistical analyses were performed with the *R* version 3.6.1 (*R* Core Team, 2019) with RStudio and GraphPad Prism 8.0 (*GraphPad Software*, San Diego, CA, United States). A *P*-value of < 0.05 (two-tailed) was considered statistically significant.

Results

WISP1 was overexpressed in BC

From the oncomine database, as illustrated in Figure 1a, we determined that WISP1 was up-regulated in BC. From the Kaplan-Meier plotter database, as presented in Figure 1b, the high expression of WISP1 was significantly associated with poor overall survival. These results indicated that the aberrant expression of WISP1 in BC was associated with unfavorable survival. qRT-PCR and Western blot analyses of cancer tissues and adjacent normal tissues from BC patients revealed that WISP1 was noticeably up-regulated in BC tissues, as shown in Figure 1c, d/e. Consistently, a significant over-expression of WISP1 was observed in BC cells (UMUC3, RT4, T24, UC9, and 5637) compared to normal cell lines (SV-HUC-1) as represented in Figure 1f/g. Collectively, these results suggested that WISP1 was significantly up-regulated in BC.

hsa-miR-29c-3p expression was negatively associated with WISP1 expression

To explore potential miRNAs related to WISP1, we used the TargetScan and identified 13 miRNAs according to their conserved sites that putatively regulate the expression of WISP1 (Figure 2a). Among these miRNAs, hsa-miR-23a-3p and hsa-miR-29b-3p were markedly up-regulated, while hsa-miR-16-5p,

hsa-miR-23b-3p, and hsa-miR-29c-3p were significantly down-regulated in BC compared to its normal adjacent tissues (Figure 2b). However, only hsa-miR-23b-3p was significantly negatively correlated with WISP1, while the others were not (Figure 2c/d/f). These results indicated that hsa-miR-29c-3p was significantly negatively correlated with WISP1 expression in BC.

WISP1 was associated with tumor size and hsa-miR-23b-3p expression

To further verify WISP1 expression in clinical specimens of BC, IHC and qRT-PCR was performed in 132 BC tissue specimens. The protein expression of WISP1 was localized both in the nucleus and cytoplasm of cells (Figure3b). The low expression of WISP1 was detected in 92 samples and 40 samples exhibited high expression of WISP1 (figure3a/b). The expression level of hsa-miR-23b-3p in the high expression group of WISP1 was evidently lower than that in the low group by Student's t-test (Figure3c). The correlation analysis of WISP1 and hsa-miR-23b-3p expression with clinical characteristics revealed that tumor size and hsa-miR-23b-3p expression were significantly associated with WISP1 expression; however, age, gender, T stage, N stage, M stage, TNM stage, and differentiation were not significantly associated with WISP1 expression (Table1). Moreover, tumor size, M stage, and WISP1 were significantly correlated with hsa-miR-23b-3p expression; however, age, gender, T Stage, N Stage, TNM stage and differentiation were not associated with hsa-miR-23b-3p expression (Table1). These findings indicated that WISP1 and hsa-miR-23b-3p might play an important role in BC.

Table2. The correlation between WISP1 expression and clinicopathological characteristics in BC

Characteristics	No. WISP1			<i>P</i> value	hsa-miR-23b-3p		
	Low	High			Low	High	<i>P</i> value
Age				0.27			0.128
<=60	78	51	27		33	45	
>60	54	41	13		15	39	
Gender				0.119			0.881
Female	74	47	27		26	48	
Male	58	45	13		22	36	
T Stage				0.239			0.42
T1-T2	68	51	17		22	46	
T3-T4	64	41	23		26	38	
N Stage				0.699			0.229
N0	61	41	20		26	35	
N1-N3	71	51	20		22	49	
M Stage				0.141			0.006
M0	55	34	21		28	27	
M1	77	58	19		20	57	
TNM Stage				0.210			0.140
I-II	45	35	10		12	33	
III-IV	87	57	30		36	51	
Differentiation				0.204			0.054
Poor and Middle	66	42	24		30	36	
Well	65	49	16		18	47	
Tumor Size				0.011			0.021
<=4 cm	60	49	11		15	45	
>4 cm	72	43	29		33	39	
hsa-miR-29c-3p				<0.001			
Low	48	17	31				
High	84	75	9				
WISP1							<0.001
Low	92				17	75	
High	40				31	9	

WISP1 and hsa-miR-23b-3p were independent prognostic factors for BC

Using Kaplan-Meier survival analysis, BLAD patients with high WISP1 expression exhibited a significantly low OS ($P = 0.011$, Figure 4a); BLAD patients with high hsa-miR-23b-3p expression exhibited a noticeably good OS ($P = 0.015$, Figure 4b). The results of the univariate analysis indicated that age (>60 vs. <=60), M stage (M1 vs. M0), TNM stage (III-IV vs. I-II), hsa-miR-29c-3p (High vs. Low), and WISP1 (High vs. Low) were critical factors affecting the overall survival in patients with BC ($P < 0.05$, Table 2).

The results of multivariate Cox regression analysis showed that TNM stage (III-IV vs. I-II) and WISP1 (High vs. Low) were independent predictors of unfavorable prognosis, while hsa-miR-29c-3p (High vs. Low) represented a predictor of favorable prognosis in patients with BC ($P < 0.05$, Table 3). Together, these results revealed that WISP1 and hsa-miR-23b-3p were independent prognostic factors of BC.

Table 3. Univariate and multivariate COX regression analyses of the prognostic factors in bladder cancer

Characteristics	Univariate Cox regression analysis			Multivariate Cox regression analysis		
	HR	95% CI	P Value	HR	95% CI	P Value
Age (>60 vs. ≤60)	2.3	1.27-4.17	0.006	1.58	0.84-2.97	0.155
Gender(Female vs. Male)	1.37	0.8-2.35	0.252			
Tumor Size (>4 cm vs. ≤4 cm)	0.84	0.49-1.44	0.518			
Differentiation (Poorly vs. Well/moderately)	0.91	0.53-1.56	0.733			
T Stage(T3-T4 vs. T1-T2)	1.25	0.73-2.14	0.413			
N Stage(N1-N3 vs. N0)	1.05	0.61-1.8	0.862			
M Stage(M1 vs. M0)	1.94	1.13-3.31	0.016	1.61	0.94-2.76	0.085
TNM Stage(III-IV vs. I-II)	2.58	1.48-4.48	0.001	2.71	1.51-4.89	0.001
hsa-miR-29c-3p (High vs. Low)	0.29	0.16-0.52	<0.001	0.46	0.22-0.96	0.038
WISP1 (High vs. Low)	2.71	1.55-4.73	<0.001	2.15	1.07-4.32	0.031

Discussion

WISP1, a cysteine-rich protein, belongs to the family of matricellular proteins involved in developmental functions and carcinogenesis. Furthermore, WISP1 polymorphisms have been recognized as a biomarker or a therapeutic target in urothelial cell carcinoma²². From the oncomine database and Kaplan-Meier plotter database, we found that WISP1 was up-regulated in BC and associated with significantly poor overall survival, suggesting a potential role of WISP1 in BC. The expression of WISP1, both at mRNA and protein levels, was evaluated in BC cell lines and tissues, and the results indicated that WISP1 was indeed overexpressed in BC. miRNAs, critical regulators of gene expression, are often dysregulated in cancer.²³⁻²⁶ Using TargetScan, we predicted 13 miRNAs, which potentially target the WISP1. However, correlation analysis revealed that only hsa-miR-23b-3p was significantly negatively correlated with WISP1 ($P=0.006$, $r=-0.633$) in BC. Notably, miR-23b-3p has also been reported as a robust normalizer for urine microRNA studies in BC²⁷. Accumulating studies have indicated that miR-23b-3p acts as a *tumor* suppressor in multiple cancer, including laryngeal squamous cell carcinoma²⁸, ovarian cancer²⁹, esophageal Carcinoma³⁰, and BC³¹; however, in abeta-treated neuroblastoma cells, *miR-23b-3p functions as an oncogenic factor*³².

Furthermore, tumor size and hsa-miR-23b-3p expression were significantly associated with WISP1, and tumor size, M stage and WISP1 were significantly correlated with hsa-miR-23b-3p expression. All these findings indicated that WISP1 and hsa-miR-23b-3p might play crucial roles in BC. The results of univariate Cox analysis revealed that age, M stage, TNM stage, hsa-miR-29c-3p, and WISP1 were critical factors affecting the survival time in patients with BC. The multivariate Cox survival analysis revealed that TNM stage and WISP1 were predictors of unfavorable prognosis, while hsa-miR-29c-3p represented a factor of favorable prognosis in patients with BC. Furthermore, BC patients with high WISP1 expression exhibited a significantly lower OS, while patients with high hsa-miR-23b-3p expression exhibited an

evidently higher OS. Taken together, these results suggested that WISP1 and hsa-miR-23b-3p were independent prognostic factors of BC.

Several potential limitations of the present study should be noted. Firstly, although validated in databases, our bioinformatics analyses with the current databases may have certain limitations and flaws. However, the evidence supporting the involvement of WISP1 and hsa-miR-23b-3p in BC remains very limited. Thus, further studies are warranted to validate with mechanistic evidence the designation of WISP1 as a potential target of hsa-miR-23b-3p for BC. Secondly, the functional mechanism underlying WISP1 overexpression in BC was not investigated; therefore, further studies are required to identify the mechanisms of WISP1 overexpression in bladder carcinogenesis.

Conclusions

In conclusion, the present study demonstrated that WISP1 is upregulated and associated with poor prognosis in BC. The miR-29c-3p negatively related with WISP1 is an independent prognostic factor in BC. The results of the present study indicated that WISP1 and miR-29c-3p may serve as novel prognostic biomarkers and potential therapeutic targets for BC.

Abbreviations

BC: bladder cancer; IHC: immunohistochemistry; WISP1: WNT1-inducible signaling pathway protein 1; miRNAs: MicroRNAs; OS: Overall survival; qRT-PCR: Quantitative real-time polymerase chain reaction; SD: Standard deviation

Declarations

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Author's contributions

Shao JF designed the research; Zhang LH, Zhou HY, Wang Z, and Zhu M performed the research; Shao JF and Xu ZQ contributed new reagents/analytic tools; Zhou HY and Wang Z analyzed the data; Zhang LH wrote the paper.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Institutional Review Board of Wuxi People's Hospital. Written informed consents were obtained from each patient.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest in this work.

Author details

¹Department of urology, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi 214023, Jiangsu Province, China

²Department of Geriatrics, Wuxi People's Hospital Affiliated to Nanjing Medical University, Wuxi 214023, Jiangsu Province, China

³Department of Gastrointestinal Surgery, Zhongshan Hospital affiliated with Xiamen University, Xiamen361004, Fujian Province, China

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Figures

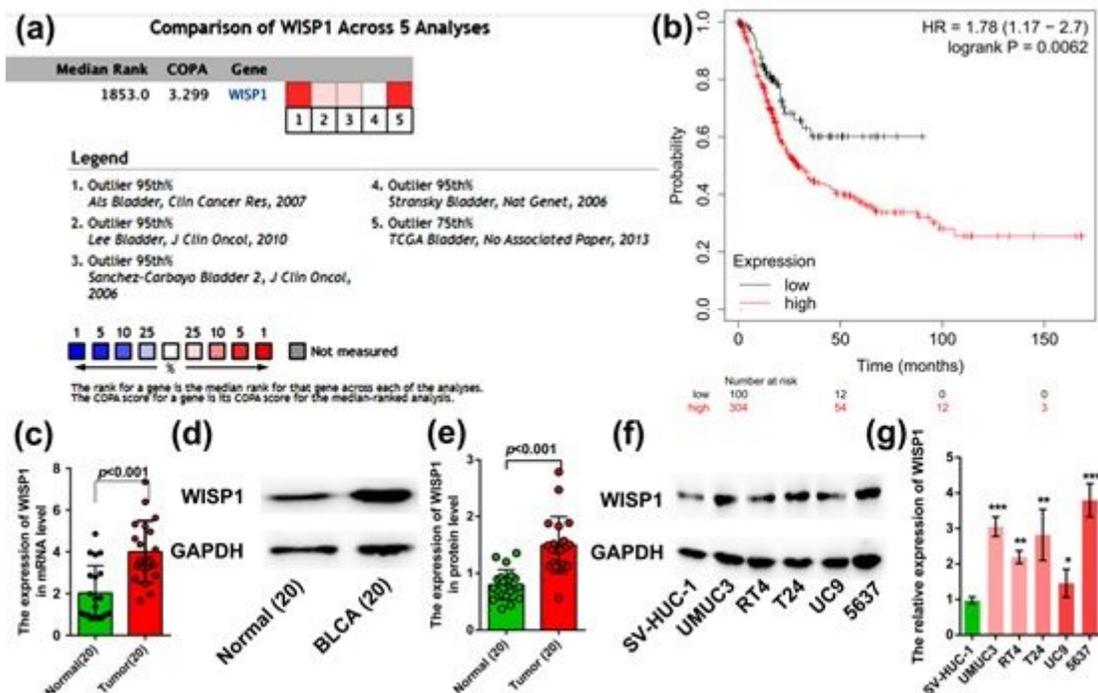


Figure 1

WISP1 overexpression in BC (a) The expression of WISP1 in BC using oncomine database; (b) Association of WISP1 expression with overall survival in BC using Kaplan-Meier plotter database; (c) WISP1 mRNA expression in BC tissues and adjacent normal tissues by qRT-PCR; (d) Western blot analysis of BC tissues and adjacent normal tissues; (e) Results of the protein expression of WISP1 in BC tissues and adjacent normal tissues by Western blot assay; (f) Results of the protein expression of WISP1 in normal and BC cell lines by Western blot assay; (g) Statistical analyses of the protein expression of WISP1 in normal and BC cell lines by Western blot assay. *** represented $P < 0.001$; ** represented $P < 0.01$; * represented $P < 0.05$.

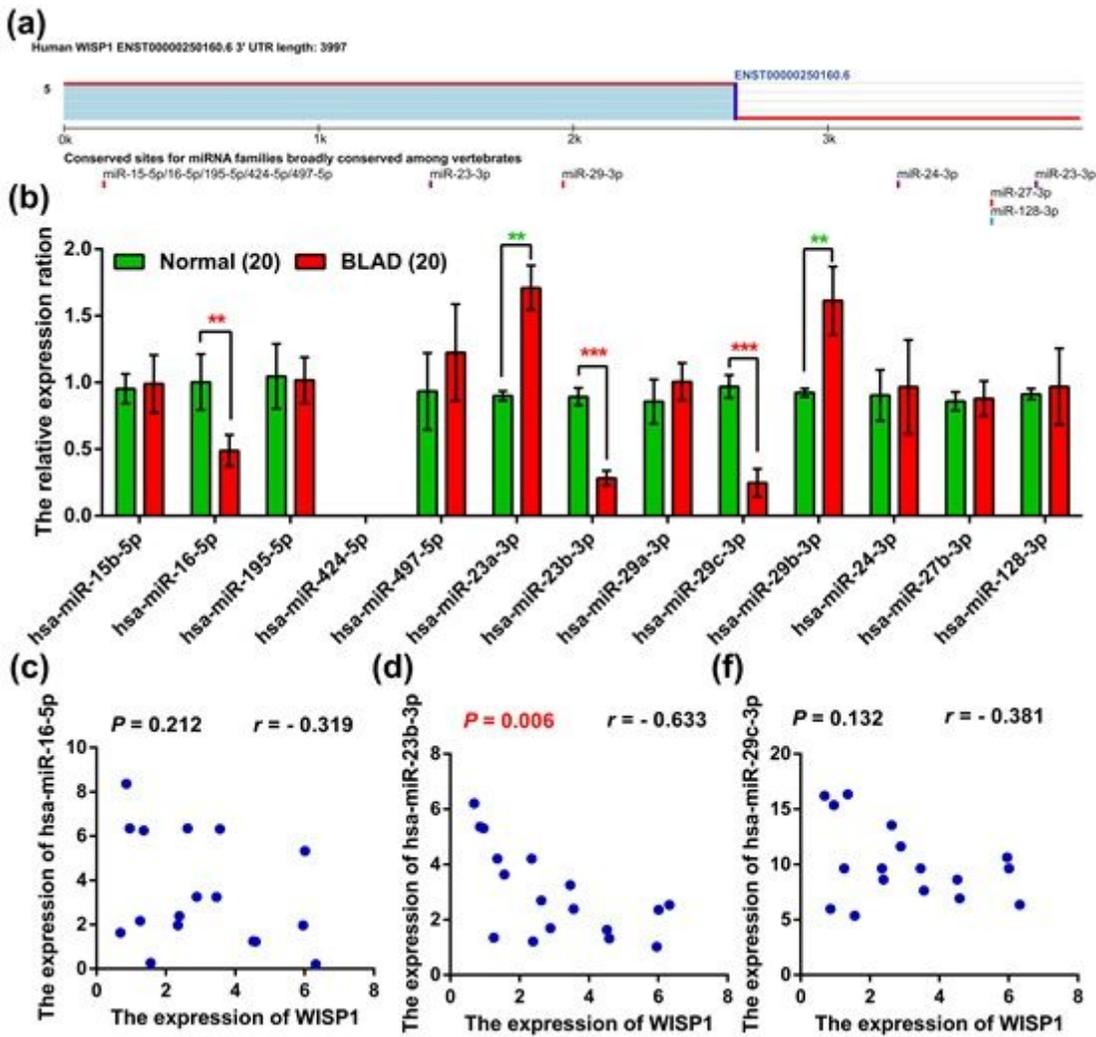


Figure 2

The miRNAs expression was associated with WISP1 in BC (a) Conserved target sites of miRNA families for WISP1 using TargetScan analysis; (b) The expression of related miRNAs in normal and BC tissues; (c, d, f) The related expression of WISP1. *** represented $P < 0.001$; ** represented $P < 0.01$.

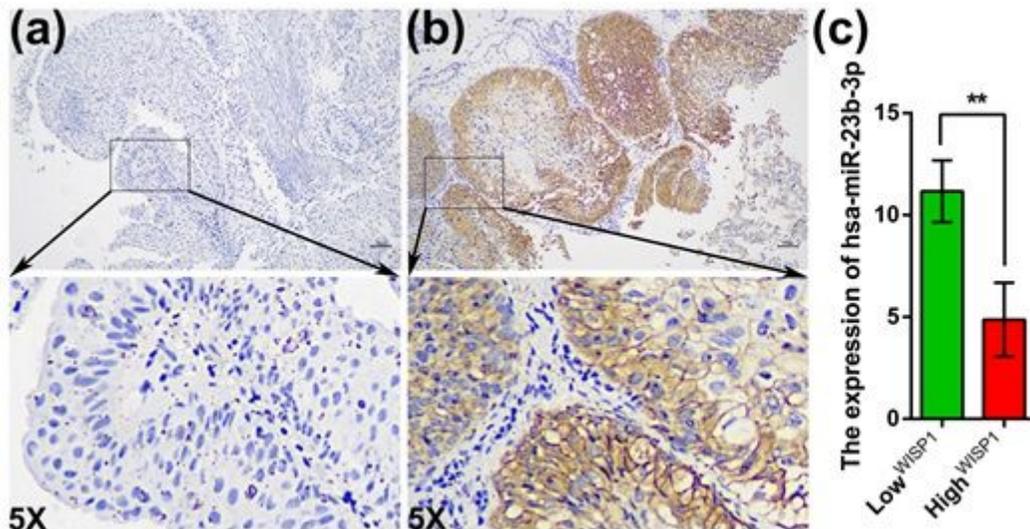


Figure 3

The correlation between WISP1 and hsa-miR-23b-3p expression (a) The low expression of WISP1; (b) The high expression of WISP1; (c) The expression of hsa-miR-23b-3p in the groups of low and high expression of WISP1. 5X indicates magnification of 5X.

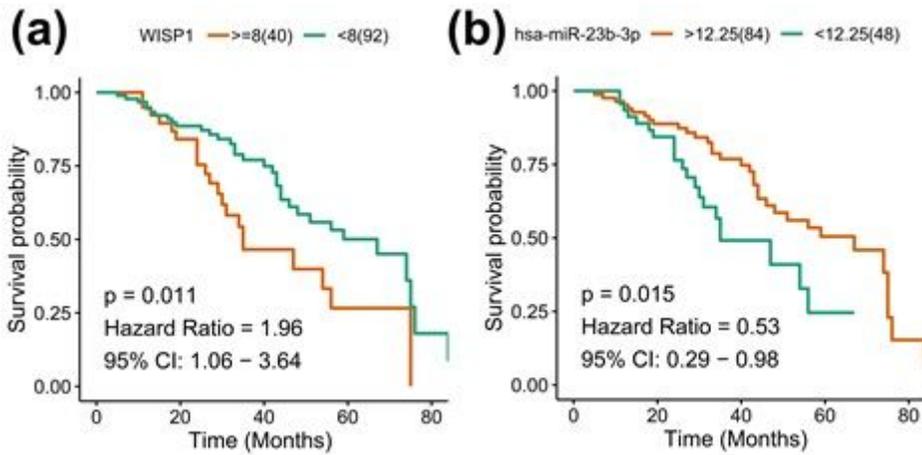


Figure 4

WISP1 and hsa-miR-23b-3p associated with overall survival of bladder cancer (a) Kaplan-Meier survival curves for WISP1 expression; (b) Kaplan-Meier survival curves for hsa-miR-23b-3p expression.