

RESEARCH ARTICLE

Expression and Significance of the Wip1 Proto-oncogene in Colorectal Cancer

Zong-Tao Li¹, Liu Zhang^{1,2*}, Xiao-Zeng Gao¹, Xiao-Hua Jiang¹, Li-Qian Sun¹

Abstract

Aim: To investigate the level of expression of proto-oncogene Wip1 and its physiological significance in colorectal cancer. **Methods:** Immunohistochemistry, semi-quantitative RT-PCR, and Western blotting were used to analyze Wip1 mRNA and protein expression in 120 cases of colorectal cancer and normal tissues to study relationships with clinical symptoms and disease prognosis. **Results:** The level of Wip1 protein expression was found to be significantly higher in colorectal cancer tissues (85% (102/120)) than in normal tissues (30% (36/120)) ($P < 0.05$). The relative amount of Wip1 protein in colorectal cancer tissue was also found to be significantly higher ($P < 0.05$) than in normal tissues (1.060 ± 0.02 and 0.640 ± 0.023 , respectively). Semi-quantitative RT-PCR showed average Wip1 mRNA expression levels to be 1.113 ± 0.018 and 0.658 ± 0.036 for colorectal cancer tissue and adjacent normal tissue ($P < 0.05$). The level of Wip1 protein expression was not correlated with age, gender, or tumor site, but appeared linked with lymph node metastasis, Dukes stage, histological grade, and liver metastasis. Individuals with high and low levels of Wip1 expression showed statistically significant differences in the five-year overall survival and recurrence-free survival rates ($P < 0.05$). **Conclusion:** Wip1 mRNA and protein are highly expressed in colorectal cancers and may be associated with colorectal cancer development and progression.

Keywords: Wip1 - colorectal cancer - immunohistochemistry - reverse transcription - polymerase chain reaction

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Introduction

Over the past 30 years, the incidence and mortality of colorectal cancer have increased significantly. Early diagnosis, degree of malignancy, and prognosis of colorectal cancer have become a hot field in biomedical research. In 1997, Fiscella et al. first discovered Wip1 through genetic screening (Fiscella et al., 1997). In 2002, Bulavin et al. identified Wip1 as a new proto-oncogene (Bulavin et al., 2002). Currently, the correlation between Wip1 gene expression and colorectal cancer has not yet been reported. In this study, we used immunohistochemistry, Western blot analysis, and semi-quantitative RT-PCR to study Wip1 expression in colorectal cancer tissues and normal tissues to explore the role of Wip1 in the development of colorectal cancer.

Materials and Methods

Clinical Data

From January 2002 to January 2007, 120 patients with colorectal cancer underwent colorectal resection in Tangshan work's Hospital. There were 66 males and 54 females, aged from 29 to 75 years old, with a median age of 54 years. All patients did not have any neoadjuvant therapies. The fresh specimens of tumor tissue or adjacent

normal epithelium 5 cm apart from the tumor edge were immediately taken after the surgery, one was fixed in 4% paraformaldehyde solution, then embedded in paraffin for immunohistochemistry, and the other one was stored in liquid nitrogen for RT-PCR and Western blot assay. Patients were staged according to the UICC cancer staging criteria for colorectal cancer (5th edition, 1997). Among 120 cases of colorectal cancer: 68 located at colon, 52 in the rectal. Eighty patients demonstrated no lymph node metastasis (N0), whereas forty patients with identified lymph nodes involvement (N+). As for the Dukes stages, 32 cases had A stage and 88 had a B to C stages. The grades of differentiation were 48 with Grade I (well differentiated) and 72 with Grade II or III (moderately to poorly differentiated). There were 31 cases where had liver metastasis and 89 cases had not liver metastasis.

Main Reagents

Rabbit anti-human WIP1 monoclonal antibody (Epitomics Inc.). β -actin mouse anti-human monoclonal antibody (Santa Cruz). immunohistochemistry kit (Zhongshan Goldenbridge Biotechnology Co., Ltd.). RNATrizol Extraction Kit (Beijing Solarbio). Superscript III reverse transcriptase kit (Invitrogen Corporation). TaqDNA polymerase, OligodT, dNTPs, reverse transcriptase, RNases inhibitor (Fermentas Inc.).

¹Department of Surgery, Hebei Medical University, Shijiazhuang, ²Department of Surgery, The Affiliated Hospital of Hebei United University, Tangshan, China *For correspondence: zhliu130@sohu.com

Immunohistochemistry

A 4 µm section was prepared from paraffin-embedded block and dehydrated, then incubated in 3% hydrogen peroxide for 10min to block endogenous peroxidase, followed by using trypsin for repair of 20 min; 10% goat serum was introduced at room temperature for closure of 20 min, and WIP1 antibody (1: 200) was left in the wet box at 4 °C refrigerator for overnight. Then the secondary and third antibodies were dropped into the wet box at room temperature for incubation of 20 min, respectively; DAB staining was again visualized by the hematoxylin stain, and then came to normal dehydration with the coverslip sealed. Results found: two pathologists without knowing patients' information were responsible for assessing the results. Regarding cell counting under microscope (400X), 5 fields were randomly selected, and 3 slides for each specimen were counted. WIP1 expression was determined based on the percentage of positive cells, combined with the staining intensity. The percentage of positive cells was divided into four levels: 0 point: ≤ 5% of positive cells, 1 point: 5% ~ 25%, 2 points: 25% ~ 50%, and 3 points: > 50% of positive cells. The intensity of staining was classified as: 0 points: no staining, 1 point: weak staining (light yellow); 2 points: moderate staining (brown); and 3 points: strong staining (yellowish- brown). The final score of WIP1 expression was the product of the WIP1 expression rate and intensity, graded as 0 for negative, + for 1-3 points, ++ for 4-6 points, and +++ for 7-9 points. As for the negative control, the primary antibody was replaced with PBS.

Semi-Quantitative RT-PCR

Total RNA extraction: specimen was removed from the liquid nitrogen, and the total RNA was extracted according to the instructions on Trizol reagent. 2µg total RNA was taken for synthesis of cDNA according to the operating requirements of Superscript III Reverse Transcriptase Kit Instructions in 20ul reaction system; Reaction conditions: denaturation at 65°C for 5min, and RT at 50°C for 50 min. PCR amplification of WIP1 gene upstream primer: 5'-TTCTCGCTTGTCACCTTGCC-3' downstream primer: 5'-CCAA ACTACACGATTCACCCC-3'', amplified fragment length of 318 bp; β-actin gene upstream primer: 5'-CACCCGCGAGTACAACCTTC-3', downstream primer: 5'-CCCATACCCACCATCACACC-3-3', amplified fragment length of 207 bp. PCR reaction 50ul include: 5 ul 10 × PCR buffer, 1 ul 10 mmol dNTP, 0.5 ul TaqDNA polymerase, the upstream and downstream primers 2 ul, respectively, 2 ul template cDNA, plus ddH₂O, was complemented to 50ul. Reaction conditions: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 45 s, a total of 35 cycles, placed at -20°C. PCR product detection and its semi-quantitative analysis: after the product underwent gel electrophoresis in 1.5% agarose, the gel imaging system combined with Multi Gauge V 3.1 was used for optical density analysis of the results.

Western Blot

Approximately 100 mg specimen was taken out of liquid nitrogen, and added with 1ml ice-cold protein lysis

buffer. Glass homogenizer was used for homogenation, and ultrasonic crusher machine for ultrasound process in 10 s × 4 with an interval of 15 s. All operations were completed on the ice. At 4°C, 12000 r/min centrifugation lasted for 20 min, and then the supernatant was taken for backup at -20°C. After the detection of protein concentration with BCA Protein Assay Kit, each hole was given a sample amount of 50ug for SDS-PAGE electrophoresis. Regulator power for ice bath was transferred to nitrocellulose membrane, followed by closure for 2 h with 5% skim milk. Subsequent to Anti-1 overnight incubation at 4°C (WIP1 1:500, β-actin 1:500), it was added with HRP labeled goat anti-rabbit IgG (1:5000) for room temperature incubation of 1 h. The next steps were ECL chemiluminescence, FUJI Mini-4000 image scanning, and application of the LabWorks 4.5 software for quantitative analysis of Western bands.

Statistical analysis

Between colorectal cancer tissues and its adjacent normal tissues, the categorical data were expressed as exact counting (%) and analyzed by means of Wilcoxon signed- rank test and paired Student's t-test. Parametric data were analyzed using chi-square test. A *P*-value < 0.05 was considered statistically significant. The statistical analysis was performed by SPSS 13.0 Windows.

Results

Immunohistochemical Wip1 staining of colorectal cancer and normal colorectal tissues

In normal colorectal tissues, Wip1 staining was negative or weak. In colorectal cancer tissues, Wip1 staining ranged from light yellow to brown. Statistically, Wip1 was expressed in 85% (102/120) of colorectal cancer tissues, which was higher than the 30% (36/120) in normal tissues. The difference was statistically significant (*P* < 0.05) (Table 1, Figure 1).

Western blot analysis of Wip1 protein expression in colorectal cancer and normal colorectal tissues

The relative amount of Wip1 protein in colorectal

Table 1. Expressions of WIP1 in Colorectal Cancer Tissue and in Normal Colorectal Tissue

Group	Case	Expression of WIP1 Protein				Z	P
		-	+	++	+++		
Cancer Tissue	120	18	18	47	37	-6.967	0.000
Normal Tissue	120	84	21	15	0		

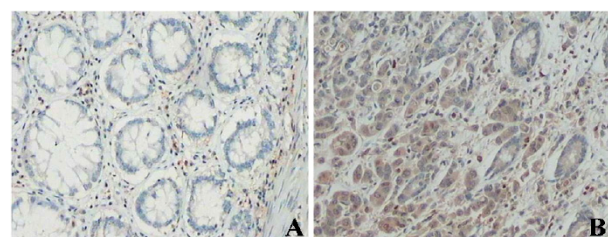
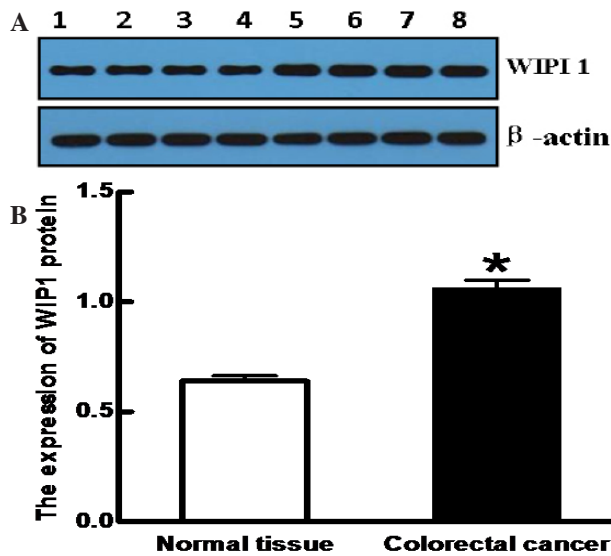
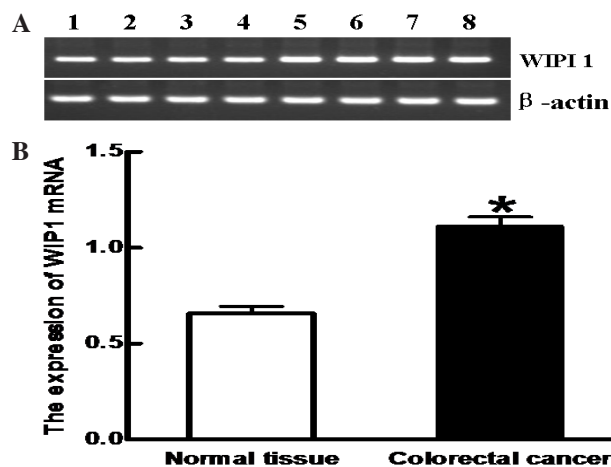


Figure 1. Expression of WIP Protein in Colorectal Cancer Tissue and in Normal Colorectal Tissue (A and B for SP×200; A for normal colorectal tissue, B for colorectal cancer tissue)

Table 2. Expressions of WIP in Colorectal Cancer Tissue and in Normal Colorectal Tissue

Group	Case	Expression of WIP1 mRNA			
		Normal tissue	Cancer tissue	t	P
Wip1 Protein Expression	120	0.640±0.023	1.060±0.020	151.164	0.000
Wip1 mRNA Expression	120	0.658±0.036	1.113±0.018	124.508	0.000

**Figure 2. Expression of WIP Protein in Colorectal Cancer Tissue and in Normal Colorectal Tissue.** 1 to 4 for normal colorectal tissue, 5 to 8 for colorectal cancer tissue. One asterisk indicates statistical significance ($P < 0.05$)**Figure 3. Expression of WIP mRNA in Colorectal Cancer Tissue and in Normal Colorectal Tissue.** 1 to 4 for normal colorectal tissue, 5 to 8 for colorectal cancer tissue. One asterisk indicates statistical significance ($P < 0.05$)

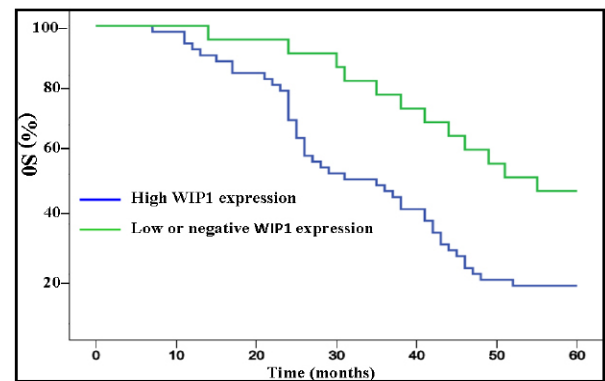
cancer tissues was found to be 1.060 ± 0.02 in colorectal cancer tissues and 0.640 ± 0.023 in normal tissues, respectively. The difference was statistically significant ($P < 0.05$) (Table 2, Figures 2A, 2B).

Semi-quantitative RT-PCR detection of Wip1 mRNA expression in colorectal cancer and normal colorectal tissues

The level of Wip1 mRNA in colorectal cancer tissues was found to be higher than in normal tissues, 1.113 ± 0.018 and 0.658 ± 0.036 , respectively. The difference was found to be statistically significant ($P < 0.05$) (Table 2, Figures 3A, 3B).

Table 3. Relation Between WIP1 Expression and Clinic Characteristics in Colorectal Cancer Tissue

Group	Case	Expression of WIP1 Protein		χ^2	P
		-~+	++~+++		
Age					
≤50	52	14	38	0.414	0.520
>50	68	22	46		
Sex					
male	66	24	42	2.828	0.093
female	54	12	42		
Lesion site					
colon	68	20	48	0.026	0.872
rectal	52	16	36		
Lymph node metastasis					
N+	40	7	33	4.464	0.035
N0	80	29	51		
Dukes stages					
A	32	16	16	8.312	0.004
B~C	88	20	68		
Histological Grade					
I	48	21	27	7.202	0.007
II~III	72	15	57		
Liver metastasis					
Yes	31	4	27	5.818	0.016
No	89	32	57		

**Figure 4. Kaplan-Meier Analyses for Overall Survival (OS) According to the WIP Expression in Colorectal Cancer**

Wip1 expression and clinical factors in colorectal cancer

The level of Wip1 expression was not found to be correlated with age, gender, or tumor site, but it was found to be correlated with the rate of lymph node metastasis, Dukes stage, histological grade, and rate of liver metastasis (Table 3).

Wip1 expression and prognosis

Survival analysis was performed in all the patients and follow-up data were collected. All patient follow-ups ended in May 2012 after a revisit time of 60 months. Among all cases, 64 were still alive at this time and 56 were dead. A survival curve was drawn. Patients were divided into two groups according to Wip1 expression level. There were 84 individuals with high levels of Wip1 expression, among whom 40 were still alive and 44 were dead. The survival rate was 47.6%. There were 36 individuals with low levels of Wip1 expression, among whom 24 were still alive and 12 were dead. The survival rate was 66.7%. Patients with low levels of Wip1 expression had significantly higher 5-year survival rates than those with

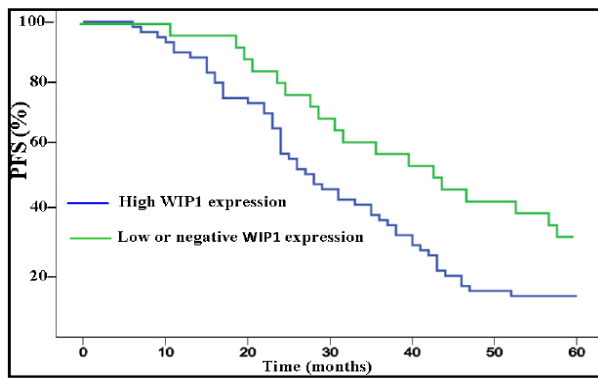


Figure 5. Kaplan-Meier Analyses for Progression Free Survival (PFS) According to the WIP Expression in Colorectal Cancer

high levels of Wip1 expression group ($P < 0.05$) (Figure 4). During the 60 months of follow-up, 48 cases were non-recurrent and 72 cases were recurrent. A survival curve was drawn. Among the 84 individuals with high levels of Wip1 expression, 30 cases were non-recurrent and 54 were recurrent, producing a non-recurrence ratio of 35.7%. Among the 36 individuals with low levels of Wip1 expression, 18 cases were non-recurrent and 18 were recurrent, producing a non-recurrence ratio of 50.0% ($P < 0.05$) (Figure 5).

Discussion

Colorectal cancer is a common malignant tumor of the gastrointestinal tract. In recent years, its global incidence and mortality have increased dramatically, exceeding those of gastric and esophageal cancer (Wan, 2009). After surgery, patient outcomes are predicted using tumor pathological stages, but the reality is that even patients who are at the same stage at the time of prognosis can experience dramatically different outcomes (Ding et al., 2006; Aaron et al 2008). The identification of colorectal- cancer-specific biomarkers may be useful to cancer therapy and prognosis. In 1997, Fiscella and colleagues discovered Wip1 gene using genetic screening and proved that Wip1 expression could be induced by wild-type p53 (Fiscella et al., 1997). Wip1 is a member of the PP2C family. It is encoded by the PPM1D (protein phosphatase magnesium-dependent 1 delta) gene on human chromosome 17q22/q23. The Wip1 protein has a molecular weight of 61 kD, and it can be divided into two functional domains. Amino acids 1–375 form a highly conserved N-terminal phosphatase domain, and amino acids 376–605 form a less conserved domain with non-catalytic activity. Wip1 is a newly discovered proto-oncogene (Le et al., 2010).

Previous studies have shown that Wip1 is highly expressed in neuroblastoma, pancreatic cancer, lung cancer, bladder cancer, liver cancer, ovarian cancer, and breast cancer (Hirasawa et al., 2003; Saito et al., 2003; Loukopoulos et al., 2007; Wang et al., 2011). However, its expression in colorectal cancer has not yet been explored. In the present study, immunohistochemistry, Western blot analysis, and semi-quantitative RT-PCR all demonstrated that Wip1 was highly expressed in colorectal cancer, but

little to no expression was detected in normal tissue. This is consistent with the findings given above. Our study is the first to propose that Wip1 may be a biomarker of colorectal cancer. However, the tumorigenic mechanism of Wip1 is not yet known (Lu et al., 2005; Doucette et al., 2012). High levels of Wip1 expression may inhibit DNA repair by reducing the activity of ATM-dependent signaling cascades, and it may promote cell cycle progression in a manner that fosters tumorigenesis (Lu et al., 2004; Shreeram et al., 2006). Recent studies have shown that when DNA is damaged, ATM phosphorylates Chk2 and so prevents tumor initiation (Spinnler et al 2011; Park et al., 2012). Wip1 may bind to Chk2 and cause the dephosphorylation and inactivation of Chk2, producing tumorigenesis.

This study shows that Wip1 expression is not correlated with age, gender, or tumor site. This is consistent with the majority of results from previously published literature. Fuku et al. have shown that high levels of Wip1 expression are correlated with tumor size and chk2 expression in gastric cancer (Fuku et al., 2007). Chaohui Liang, et al. have shown that Wip1 expression in pleomorphic glioblastoma is not correlated with patient gender, tumor size, or age (Liang et al., 2012). Our study was the first to demonstrate that Wip1 gene expression is correlated with lymph node metastasis, Dukes stage, the degree of tumor differentiation, the degree of liver metastasis, indicating its close correlation with metastasis of colorectal cancer. This conclusion directly contradicts that of a previous report by Ren, et al., which showed that, in papillary thyroid tumors, Wip1 expression is not correlated with tumor histological type, lymph node metastasis, or TNM stage (Ren et al., 2012). This contradiction may be attributable to the differences in tissue types, research methods, techniques, and regulatory mechanisms.

Most scholars believe that Wip1 overexpression is closely related to prognosis (Satoh et al., 2011). Hu et al. found that, in pancreatic neuroendocrine tumors, Wip1 high-expression is closely correlated to poor prognosis (Hu et al., 2010). In a follow-up study, Robert C. et al. found that, in medulloblastoma patients, high levels of Wip1 expression were correlated with poor prognosis (Castellino et al., 2008). However, Yu et al. showed that Wip1 gene expression is not correlated with patient prognosis (Yu et al., 2007). In our study, we conducted a 5-year follow-up of patients treated for colorectal cancer. We firstly demonstrated that, among individuals with colorectal cancer, high levels of Wip1 expression had lower 5-year survival and recurrence-free survival rates than those with only low levels of Wip1 expression ($P < 0.05$). This is consistent with the conclusion that Wip1 may be a suitable prognostic factor in patient assessment. We believe that the poor prognosis of individuals with high levels of Wip1 expression may be due to their high rates of lymph node and liver metastasis.

Currently, gene therapy shows great value in the treatment of colorectal cancer, but only a few new genes have been applied to clinical treatment, and key therapeutic targets have not yet been found (Hayashi et al., 2011). Our study is the first to show high levels of Wip1 gene expression in colorectal cancer patients.

It provides a new target for colorectal cancer therapy. The mechanism or pathway by which Wip1 affects the incidence and development of colorectal cancer merits needs further investigation.

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