

RAPID COMMUNICATION

Overexpression of connective tissue growth factor WISP-1 in Chinese primary rectal cancer patients

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may play a role in rectal tumorigenesis. WISP-1 may be used as a specific clinical diagnosis and prognosis marker in rectal cancer.

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Abstract

AIM: To clarify the expression change of Wnt-induced secreted protein-1 (WISP-1) in human rectal cancer and to determine whether it is correlated with invasion and metastasis of human rectal cancer.

METHODS: Eighty-six paired samples of rectal cancer and surgically resected distant normal rectal tissue were collected and allocated into cancer group and control group respectively. WISP-1 mRNA was detected by relative quantitative real-time RT-PCR and WISP-1 protein was examined by immunohistochemical staining.

RESULTS: WISP-1 gene overexpression was found in 65% (56/86) primary rectal cancers, 2-30 times that of the level in normal matched rectal tissues ($P = 0.001$). The mRNA expression level was correlated with Duke's staging, histological differentiation grade and lymph node status. The WISP-1 protein expression was in accordance with mRNA expression level. The positive degree of immunohistochemical staining in the cancer group (1.40 ± 0.35) was different from that in control group (1.04 ± 0.08 , $P < 0.001$). Moreover, in cancer group the positive staining degree in high-level mRNA cancers (1.46 ± 0.37 , $n = 56$) was higher than that in low-level mRNA (1.28 ± 0.28 , $n = 30$, $P = 0.018$).

CONCLUSION: Aberrant levels of WISP-1 expression

INTRODUCTION

Wnt-induced secreted protein-1 (WISP-1) is a member of the connective tissue growth factor that belongs to the Cry61, CTGF and Nov (CCN) superfamily. All members of that family possess a secretory signal peptide at the NH2 terminus, indicating that they are secreted proteins. The biological properties of CCN proteins include stimulation of cell proliferation, migration, adhesion, and extracellular matrix formation. They also regulate more complex biological processes, such as angiogenesis and tumorigenesis^[1-3]. WISP-1 is strongly expressed in the fibrovascular stroma of breast tumors developing in Wnt-1 transgenic mice^[4]. Moreover, forced overexpression of WISP-1 in normal rat kidney fibroblasts was sufficient to induce their transformation, increase cellular saturation and promoted growth^[5].

WISP-1 is a potential downstream target gene of Wnt-1/APC/ β -catenin signaling pathway in colorectal cancer^[5]. Though the expression of WISP-1 was believed to increase in colon carcinoma^[4], it has not been quantitatively detected with a larger number of samples in rectal cancer alone. In this study, mRNA and protein expression of WISP-1 in pair matched rectal samples were measured by real-time RT-PCR and immunohistochemical staining, respectively, and the association between WISP-1 expression level and clinicopathologic features was further investigated. Through this work we aimed to elucidate the role of WISP-1 in the invasion and metastasis of rectal cancer and to provide a basis for its clinical diagnosis and outcome prediction.

MATERIALS AND METHODS

Patients and samples

Eighty-six paired samples of rectal cancer and distant normal rectal tissues were obtained from inpatients who underwent surgical operation from 2003 to 2004 in West China Hospital of Sichuan University. These patients did not receive any chemotherapy or radiotherapy before operation. Diagnoses and routine histopathologic examinations of frozen sections were performed at the Department of Pathology, West China Hospital. Immediately after selection of diagnostic tissue samples and dissection of the margins for diagnostic purposes, samples of tumor and tumor-free tissues were collected respectively and stored in liquid nitrogen at -80°C . Samples were fixed by formalin and embedded in paraffin wax. The following information was recorded for each patient: age, sex, Duke's stage, carcinoembryonic antigen (CEA), lymph node status and histological grade. This study was approved by the Medical Ethical Committee and all patients provided written informed consent to participate in the study.

Total RNA extraction and cDNA preparation

Total RNA was extracted from each sample using Trizol reagent (Beyozol Co., Ltd) following manufacturer's instruction. The concentration of RNA was measured by spectrophotometer. Total RNA was reverse-transcribed to cDNA with RT reagents (Takara Biotechnology Co., Ltd) according to the manufacturer's protocol. Briefly, the RT reaction was carried out in a final volume of 20 μL containing $1 \times \text{M-MLV}$ buffer, 20 units of RNase inhibitor, 2.5 $\mu\text{mol/L}$ random hexamers, 100 units of M-MLV RTase, and 5 μL of total RNA. The mixture was incubated at 20°C for 10 min and 42°C for 60 min, and reverse transcriptase was inactivated by heating at 95°C for 10 min.

Primers and probes

As shown in Table 1, specific primers and probes for WISP-1 gene and glyceraldehyde phosphate dehydrogenase (GAPDH) gene (reference gene) were designed based on sequence data from the ensemble database (<http://www.ensembl.org>). Primers and probe were placed at the junction between two exons. The primers and probes were purchased from Takara Biotechnology Co. (Dalian, China).

Real-time RT-PCR

Conditions for all PCRs were optimized on iCycler iQ (BioRad, USA) and the optimum annealing temperature was 57.5°C . The following iCycler iQ running protocol was used: denaturation (95°C , 5 min), amplification and quantification repeated 50 times (95°C for 20 s, 60.5°C for 30 s, and 72°C for 30 s). In addition, a non-template control (ddH₂O control) was analyzed for each template. All samples were amplified simultaneously in triplicate. Equation 1 was applied to calculate the relative expression ratio (R) of WISP-1 gene, which was based on PCR efficiency (E) and the Ct deviation of an unknown sample versus a control. $\text{Ratio(R)} = (\text{EWISP-1})^{\Delta\text{CtWISP-1 (control-cancer)}} / (\text{EGAPDH})^{\Delta\text{CtGAPDH (control-cancer)}}$ (Equation 1)^[6], ΔCt was the

Table 1 Primers and probes

	Sequence
Target gene/WISP-1	
Forward primer	5'-CCACCGGGCCTACT-3'
Reverse primer	5'-CCACACCGACCACCTGT-3'
Probes	5'-FAM-CTATTGCGTACCTCGGGCGGTC-TAMRA-3'
Reference gene/GAPDH	
Forward primer	5'-CCTCAAGATCATCAGCAAT-3'
Reverse primer	5'-CCATCCACAGTCTCTGGGT-3'
Probes	5'-FAM-ACCACAGTCCATGCCATCAC-TAMRA-3'

Ct deviation of WISP-1 and GAPDH, PCR efficiency (E) was calculated according to $E = 10^{[-1/\text{slope}]}$ ^[7]. The ratio over 1 represented an up-regulation of mRNA expression of WISP-1 in rectal cancer.

Immunohistochemistry

Sections were dewaxed in xylene and rehydrated in alcohol. The endogenous peroxidase activity was suppressed by 3% hydrogen peroxide for 15 min. After rinsing twice in phosphate-buffered saline (PBS), antigen retrieval was performed by immersing the sections in 10 mmol/L sodium citrate buffer (pH = 6.0) and heated for 15 min in a microwave oven. Non-specific binding was blocked by incubation with 3% bovine serum albumin (BSA) for 40 min. The sections were treated for 16 h with rabbit antihuman polyclonal immunoglobulin G antibodies of WISP-1 (Santa Cruz, SC-25441, CA) according to the manufacturer's recommended concentration (1:50). PBS was used as a negative control. After washed three times in PBS, the sections were treated with biotinylated goat anti-rabbit immunoglobulin (Jin Mei Biotech Co., Ltd) for 40 min and then by horseradish peroxidase-streptavidin complex (Jin Mei Biotech Co., Ltd) for 30 min. The slides were then washed three times in PBS and incubated in DAB for 2 min. The slides were rinsed gently with distilled water and counterstained with haematoxylin for 30 s. The slides were dehydrated in alcohol prior to mounting. Images were collected by Olympus DD70 BX51 (Olympus, Japan) and analyzed by IMAGE-PRO plus 4.1 software (Media Cybernetics, USA). Eight visual fields in each section were randomly selected and the mean value of relative optical density (OD) was measured and calculated by taking the OD of background as 1. The extent of immunohistochemical staining was categorized as positive (1-1.5) and strongly positive (over 1.5).

Statistical analysis

The relative expression analysis of the target gene was performed using a new software, named REST-XL[®] (relative expression software tool-XL, available at <http://www.wzw.tum.de/gene-quantification/>) for group-wise comparison in real-time PCR^[8]. Using SPSS 12.0 software, a Chi-square test (χ^2) test was performed to analyze the correlations of WISP-1 mRNA or protein expression levels with clinical and pathological parameters. $P < 0.05$ was considered statistically significant.

Table 2 Output of randomization test in two groups

	Reference gene (GAPDH)	Target gene (WISP-1)
Efficiency	1.61	1.85
Control group		
<i>n</i>	86	86
Mean	25.43	38.91
Standard deviation	15.86	58.89
CV (%)	62.35	151.36
Sample group		
<i>n</i>	86	86
Mean	24.74	35.04
Standard deviation	14.3	37.1
CV (%)	57.8	105.88

RESULTS

Patients and clinicopathologic features

Patients' age ranged from 41 to 86 (55.8 ± 13.8 ; mean \pm SD) years, 44 were males and 42 females. Carcinoma cells could be observed in all rectal cancers under a light microscope. Samples at Duke's Staging A, B, C and D were 15, 32, 25 and 14, respectively. There were 33 papillary adenocarcinomas, 37 tubular adenocarcinomas, 8 mucoid adenocarcinomas, 7 signet-ring cell carcinomas and one squamous carcinoma by histological classification. Histologically, 18, 63 and 5 were poorly, moderately and well differentiated, respectively.

mRNA expression of WISP-1 in rectal cancer tissues

RT-PCR products had the desired length (WISP-1, 80 bp; GAPDH, 141 bp). No primer-dimers were generated during the applied 50 real-time PCR amplification cycles. Real-Time RT-PCR amplification efficiencies were calculated from the given slopes in FTC-2000 software and REST[®]. Investigated transcripts showed different real-time PCR efficiency in the investigated range with high linearity (Pearson correlation coefficient $r = 1$). The real-time PCR efficiency of WISP-1 and GAPDH is 1.61 and 1.85, respectively.

The relative ratio (R) is presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the control. Therefore, an R value greater than 1.0 was considered to be an overexpression of WISP-1 gene. Among the 86 rectal cancer RNA samples tested, WISP-1 gene overexpression was found in 65% (56 of 86) primary rectal tumors, being 2-30 times that of the level in normal matched rectal tissues. Major differences in the amount of WISP-1 mRNA were observed: 17 tumors showed an expression level 1-5 times, 6 tumors 5-10 times, and 33 tumors more than 10 times that of the normal rectal tissue RNA. REST[®] analysis showed mRNA expression of WISP-1 is up-regulated in cancer group in comparison with control group by the factor 7.744, and target gene-cancer group is significantly different from target gene-control group ($P = 0.001$). Randomization data are shown in Table 2.

Correlation between WISP-1 mRNA levels and clinical and pathological features

A strongly significant association existed between Duke's

Table 3 Relationship between mRNA WISP-1 status and standard clinical, pathological factors

Factors	<i>n</i>	WISP-1 mRNA expression		<i>P</i>
		-	+	
Age(yr)				
< 60	48	16	32	0.735 ¹
≥ 60	38	14	24	
Sex				
Female	42	11	31	0.098 ¹
Male	44	19	25	
Histological differentiation				
Poor	18	2	16	
Moderate	63	25	38	0.039 ²
Well	5	3	2	
CEA ³ (ng/mL)				
≤ 3.4	20	9	11	0.126 ¹
> 3.4	22	5	17	
Lymph node status				
Negative	51	24	27	0.004 ¹
Positive	35	6	29	
Duke's staging ⁴				
A + B	47	22	25	0.011 ¹
C + D	39	8	31	
Histological type				
Tubular adenocarcinoma	37	17	20	0.081 ¹
Papillary adenocarcinoma	33	22	11	

¹ χ^2 test; ²Non-parametric test (Mann-Whitney); ³Tumor marker. Information is available for 42 patients; ⁴Duke's staging A and B were combined, and C and D were combined.

staging, lymph node status, and histological grade versus WISP-1 mRNA as shown in Table 3.

Immunohistological expression of WISP-1 in rectal cancer

The representative results are shown in Figure 1. The cytoplasm of rectal cancer cells were positively stained, but the stromal components were not. Protein expression of WISP-1 in normal rectal tissues was very weak. There was no detectable immunoreactivity in PBS control slide. The positive degree of immunohistochemical staining in the cancer group (1.40 ± 0.35) was different from that in the normal group (1.04 ± 0.08 , $P < 0.001$). Moreover, in the cancer group, the positive staining degree in high-level mRNA cancers (1.46 ± 0.37 , $n = 56$) was higher than that in low-level mRNA ones (1.28 ± 0.28 , $n = 30$, $P = 0.018$). WISP-1 protein expression was also correlated with Duke's staging, lymph node metastasis and histological grade, which is correlated with mRNA expression.

DISCUSSION

Colorectal carcinoma is the third most common malignancy in the world^[9]. Although surgical resection has been shown to be effective for localized disease, current treatment regimens are ineffective for metastatic disease, and thus new treatment strategies are required. A better understanding of the molecular events in colorectal carcinoma is pivotal in the development of novel treatment strategies. WISP-1 is a member of the connective tissue growth factor identified to be a Wnt-1 and β -catenin regulated protein^[5]. Overexpression of WISP-1 induced morphological transformation, increased

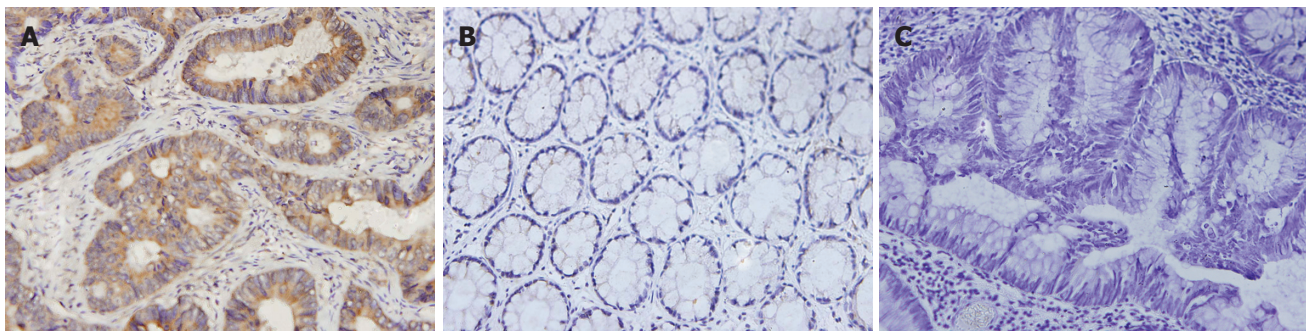


Figure 1 Representative slides showing the immunohistochemical staining of WISP-1 in rectal tissues ($\times 400$). **A:** strong WISP-1 immunoreactivity was observed in the cytoplasm of rectal carcinoma tissue; **B:** weak WISP-1 immunoreactivity was also detected in the cytoplasm of apparently normal adjacent rectal tissues; **C:** negative control slide with PBS was not positively stained in rectal carcinoma tissues.

cellular saturation, promoted growth in normal rat kidney fibroblasts, and induced tumor formation in nude mice^[5]. WISP-1 was reported to be able to attenuate p53-mediated apoptosis in response to DNA damage through activation of Akt kinase^[10]. Although WISP-1 transcript has been overexpressed in 84% of human colon adenocarcinomas and 46% of primary breast tumors^[4,11], WISP-1 expression has not been quantitatively detected in a considerable number of rectal cancers till now. In this study, quantitative real-time RT-PCR and immunohistochemistry were used to detect WISP-1 expression in pure rectal cancer and normal rectal tissues.

Although traditional PCR and RT-PCR may also yield relatively precise results, they not only need a rather large amount of template for the analysis of a sample, but also require post-PCR analysis which may create risks in cross-contamination of samples with PCR products^[12]. However, real-time RT-PCR can overcome such disadvantages and makes RNA quantifications much more precise and reproducible, because it is based on Ct values established in the early exponential phase of the PCR reaction rather than endpoint measurement of the amount of accumulated PCR product. Real-time RT-PCR has good intra-assay and inter-assay reproducibility and yields statistical confidence values, it therefore, has a high level of interlaboratory standardization and fits interlaboratory comparison^[13,14]. In this study, we utilized a new software tool, REST-XL^{®[8]}, to test the group difference for significance with a newly developed randomization test^[15,16]. The quantities of WISP-1 mRNA derived from ratios and variances can be high, normal distributions could not be expected, and it remains unclear how a parametric test could be best constructed. A randomization test, which makes no assumptions about the distribution of observations in populations, is a useful alternative to more standard parametric tests for analyzing experimental data. It is more flexible than non-parametric tests based on ranks and does not reduce in power compared to parametric test^[15]. Therefore, a randomization test with a pair-wise reallocation was seen as the most appropriate approach for this application.

The result suggested that the mRNA expression level of WISP-1 was up-regulated in rectal cancer. WISP-1 mRNA expression was also found to be significantly

associated with Duke's staging, lymph node status, and histological grade, which confirmed the role of WISP-1 in the progression of rectal cancer. The same results were found in previous reports on mRNA expression of WISP-1 in colon cancers and breast cancer^[4,11]. As shown in this study, the expression ratio over 1 in the lymph node positive group (82.9%) was higher than that in the lymph node negative group (52.9%), which suggests that WISP-1 may facilitate the lymph metastasis of rectal cancer. We also found that WISP-1 was significantly associated with poor histological differentiation, which was inconsistent with the report in colorectal cancer by Khor *et al.*^[17]. This contradiction may be related to sampling errors or insufficient experimental samples. However, our study is the largest study published to date, with 86 human rectal cancers. For Duke's staging, only 25 of 47 (53.2%) cases with stage A or B overexpressed WISP-1 compared with 31 of 39 (79.5%) samples from patients with stage C or D rectal cancers, which is in agreement with the report of Xie *et al.*^[11] in breast cancer. The Duke's staging represented the rectal cancer prognosis. The later the stage, the worse the prognosis was, so the WISP-1 may be selected as a prognosis factor. The above results indicated that overexpression of WISP-1 might play an essential role in invasion and metastasis of rectal cancer. Thus, WISP-1 mRNA expression assay could be used in the evaluation of clinical diagnosis and prognosis of rectal cancer.

WISP-1 protein expression in rectal cancer cells in this study showed moderate to strong diffuse cytoplasmic staining, and weak to non-WISP-1 immunoreactivity in the cytoplasm of apparently normal adjacent rectal tissues, which was usually most pronounced at the invasive front of the colorectal cancer^[18]. Our study also found that WISP-1 protein expression in rectal cancer was consistent with its mRNA expression and associated with Duke's staging, lymph node status and histological grade. For histological differentiation, Khor *et al.*^[17] reported that WISP-1 protein expression was associated with well-differentiated colorectal carcinoma tissues. With respect to the mechanisms underlying the difference of WISP-1 expression between rectal cancer and colon cancer, gene mutation may play a key role in different cancer cells and histological specificity after transcription of WISP-1 mRNA. By analyzing the mRNA and protein expression

of WISP-1, it tempts us to speculate that WISP-1 plays an important role in rectal carcinogenesis. But the exact mechanisms about the regulation of WISP-1 expression in rectal tumorigenesis have been unknown. A recent report by Su *et al*^[10] demonstrated that WISP-1 activates the antiapoptotic Akt signaling pathway, inhibits the mitochondrial release of cytochrome c, up-regulates antiapoptotic protein Bcl-XL, and therefore prevents cells from undergoing p53-mediated apoptosis in response to DNA damage. To better understand the regulatory mechanisms of WISP-1 expression, further studies about effects of signaling pathway on WISP-1 expression in rectal cancer are underway.

In summary, we showed for the first time, that the involvement of the WISP-1 overexpression in rectal tumorigenesis may play a role in the invasion and metastasis of human rectal cancer. WISP-1 may be selected as a clinical diagnosis and prognosis index in rectal cancer, which may also serve as a potential therapeutic target for the development of new treatment regimens for rectal cancer.

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