c-Jun suppresses the expression of WNT inhibitory factor 1 through transcriptional regulation and interaction with DNA methyltransferase 1 in gallbladder cancer

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Abstract. WNT inhibitory factor 1 (*WIF-1*) is involved in the tumorigenicity and progression of several types of tumor, which has been attributed to aberrant hypermethylation of its promoter. However, the role of *WIF-1* in the pathogenesis of gallbladder cancer (GBC) remains to be fully elucidated, and the data available are insufficient to identify the upstream molecular mechanisms involved. In the present study, the methylation status of the *WIF-1* promoter was investigated using methylation-specific polymerase chain reaction (PCR) and bisulfate sequencing PCR in GBC cells. Immunohistochemistry, reverse transcription-quantitative PCR and western blotting were used to analyze the expression of WIF-1 and c-Jun. In addition, a co-immunoprecipitation assay was designed to determine the DNA methyltransferase that was implicated in *WIF-1* methylation. The results revealed that the expression of *WIF-1* was low in GBC, and that this was caused by aberrant DNA hypermethylation. However, there were no significant correlations between the expression of *WIF-1* and certain key clinicopathological characteristics of GCB. Subsequently, a negative correlation was found between the protein expression of c-Jun and WIF-1 in 50 GBC specimens using immunohistochemistry. The demethylation and re-expression of *WIF-1* was observed when the expression of *c-Jun* was silenced. Finally, it was found that the knockdown of *c-Jun* downregulated the expression of DNA methyltransferase 1 (DNMT1) and that c-Jun interacted with DNMT1. Taken together, the present study suggested that *c-Jun* suppressed the expression of *WIF-1* through transcriptional regulation and interaction with DNMT1 in GBC. These findings provide an alternative pathogenesis of GBC, which may be promising as a novel reference for early diagnosis or future treatment.

Introduction

Gallbladder cancer (GBC) is the most common and aggressive type of malignant tumor of the biliary tract in humans (1). The lack of markers for the timely prediction and diagnosis of GBC, and its frequently silent and rapid progression confer a poor prognosis. Statistics show that, due to failure in promptly diagnosing GBC, <10% of patients are suitable to undergo curative resection (2). Furthermore, the overall mean survival rate is only 6 months in the majority of patients, even following surgery, and the five-year survival rate is only 5% (3). To date, the elucidation of several molecular mechanisms has revealed the roles of genetic and epigenetic changes involved in the tumorigenicity and progression of human cancer. However, these factors in GBC remain to be fully elucidated.

The aberrant methylation of DNA, the most well known type of epigenetic modification, occurs at CpG islands in promoter regions and generally leads to methylation-based downregulation or silencing of gene expression. The downregulation of tumor suppressors, including *P53* (4), phosphatase and tensin homolog (5), cyclin D2 (6) and Wnt inhibitory factor-1 (*WIF-1*) (7), caused by aberrant promoter methylation is integral in the occurrence and development of several types of cancer. Modifications in methylation are mediated by DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b. DNMT1 maintains its methyltransferase activity to reestablish methylation patterns

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throughout the DNA synthesis process, whereas DNMT3a and DNMT3b act as *de novo* methyltransferases (8).

WIF-1, an antagonist of the Wnt/β-catenin pathway has been demonstrated to be an important tumor suppressor (7). As a secreted frizzled-related protein, WIF-1 can directly bind to Wnt molecules at the cell surface, thereby preventing Wnt from binding to receptors (9). It has been demonstrated that the expression of *WIF-1* is decreased in several types of cancer, including breast cancer (10), lung cancer (11) and cervical cancer (12). The low expression of *WIF-1* leads to abnormal activation of the Wnt pathway, resulting in the dysregulation of cell proliferation and differentiation, and inducing carcinogenesis in humans. There is also increasing evidence supporting the hypothesis that low expression levels of *WIF-1* can largely be attributed to aberrant hypermethylation of its promoter region (10-12). In our previous study, it was demonstrated that *WIF-1* was downregulated in GBC cell lines and tissues, which led to aberrant activation of the Wnt pathway, altering the processes of proliferation, invasion, metastasis and apoptosis; *WIF-1* was involved in the tumorigenicity and progression of GBC through these effects (13). However, the upstream molecular mechanism regulating *WIF-1* remains to be fully elucidated, particularly in GBC.

c-Jun, the first oncogenic transcription factor to be identified, is the cellular homolog of the viral oncoprotein v-Jun, which is transcriptionally activated at Ser63 and Ser73 by Janus kinase (14). Early experiments revealed that the cooperation between *c-Jun* and oncogenic RAS is involved in tumor initiation and increased invasiveness in humans (15,16). In addition, *c-Jun* has been shown to lead to loss of function of the tumor suppressor p53 (17,18). *c-Jun* is widely expressed in different human tumors, is involved in numerous cell signaling pathways, and contributes to the pathogenesis, invasion and metastasis of cancer through diverse mechanisms (19,20).

The present study provided novel evidence demonstrating that the low expression of *WIF-1* in GBC cells was caused by aberrant hypermethylation of its promoter. The results revealed a negative correlation between the protein levels of c-Jun and WIF-1 in 50 GBC specimens. It was subsequently determined that the knockdown of *c-Jun* using RNA interference restored the expression of *WIF-1*. Based on these results and previous findings, it was hypothesized that *c-Jun* affects the process of methylation, particularly that performed by major methyltransferases, which leads to hypermethylation and downregulation of the *WIF-1* gene in GBC.

Materials and methods

Specimens. A total of 50 GBC sample tissues and 20 cholecystitis tissues were obtained from patients at the Department of Surgery and Pathology of Fujian Medical University Union Hospital (Fujian, China) between 2006 and 2013. None of the GBC patients had received any preoperative chemotherapy or radiotherapy. The tissue samples were fixed in 10% buffered formalin and embedded in paraffin wax. Written consent was obtained from each patient to perform experiments on the resected specimens, and the study was approved by the ethnical committee of the Medical Faculty of Fujian Medical University in accordance with the 1975 Declaration of Helsinki.

Immunohistochemistry and evaluation. Serial 4 μm sections were obtained from the formalin-fixed and paraffin-embedded tissues. Following deparaffinization in turpentine and rehydration in an alcohol gradient, the tissue sections were incubated in 3% hydrogen peroxide for 10 min at room temperature ($\sim$25°C) to prevent the activity of endogenous peroxidases. The tissue sections were subjected to antigen retrieval through boiling in citrate buffer (pH 6.0) for 10 min in a microwave, following which they were cooled at room temperature for 45 min and washed with phosphate-buffered saline (PBS). Non-specific antigens in the sections were blocked via incubation for 20 min in 5% normal goat serum (Beyotime Institute of Biotechnology, Shanghai, China) diluted in PBS. The tissue sections were subsequently incubated in a 1:100 dilution of a rabbit polyclonal anti-human c-Jun antibody (cat. no. ab31419; Abcam, Cambridge, UK) or a 1:100 dilution of a rabbit polyclonal anti-human WIF-1 antibody (cat. no. ab186845; Abcam) overnight in humidified boxes at 4°C. Following being washed in PBS, the sections were incubated according to the instructions provided with the UltraSensitive S-P kit (Maixin-Bio, Fuzhou, China). Prior to being dehydrated and mounted, the sections were stained with 3,3'-diaminobenzidine (DAB) for 3-5 min and counterstained using hematoxylin for 15 min. PBS was substituted for the primary antibody as a negative control. Cells showing deposition of buff-colored granules in the cytoplasm and nucleus were scored as c-Jun- or WIF-1-positive. The expression levels of c-Jun or WIF-1 were semiquantitatively analyzed using the mean optical density (MOD), defined as the integral optical density/positive area, calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Cell culture and treatment. The human GBC cell lines, NOZ (Health Science Research Resources Bank, Osaka, Japan), GBC-SD (Shanghai Institutes for Biological Sciences, Shanghai, China) and SGC-996 (Tumor Cytology Research Unit, Medical College, Tongji University, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; HyClone, GE Healthcare Life Sciences, Logan, UT, USA) under 5% CO_2/95% air, in a humidified 37°C incubator.

The GBC cell lines were divided into 6-well plates at a density of 2×10^5 cells/well ($\sim$30% confluence) 12 h prior to treatment. The cells were treated with 5-aza-2'-deoxycytidine (DAC; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) dissolved in dimethyl sulfoxide at a concentration of 5.0 μM, which was replaced every 24 h for a total treatment duration of 72 h.

Cell transfection. Appropriate small interfering (si)RNA target sequences were determined based on the human *c-Jun* sequence (GenBank accession no. NM_002228.3). DNA template oligonucleotides corresponding to three siRNA sequences were designed according to the guidelines for siRNA design: P-1 sense, 5'-GGACCUUAUGGCUACAGUATT-3' and antisense, 5'-UACUGUAGCCAUAAGGUCCTT-3'; P-2 sense, 5'-ACG CAAACCUCAGCAACUUTT-3' and antisense, 5'-AAGUUG CUGAGGUUUGCGUTT-3'; and P-3 sense, 5'-GGAACAGGU GGCACAGCUUTT-3' and antisense, 5'-AAGCUGUGCCAC

Table I. Primers used for polymerase chain reaction and sequence analyses.

Gene	Forward primer	Reverse primer
WIF-1	5'-CCAGGACTAGAGGGAGAGCA-3'	5'-TCGCAGACAGGCTTTGAACA-3'
c-Jun	5'-AGGAAGCTGGAGAGAATCGC-3'	5'-GTTAGCATGAGTTGGCACCC-3'
DNMT1	5'-AGAAGTGAAGCCCGTAGAGTG-3'	5'-ATGAGATGTGATGGTGGTTTGG-3'
DNMT3a	5'-TGTAACGAAGTGAAGGAGGAGAA-3'	5'-CATCTTGCCGAGGGAGTCT-3'
DNMT3b	5'-AGAGGAGTGTGAAGCAAGGA-3'	5'-TGAGAAATGAGGGTAGCAGACT-3'
GAPDH	5'-AGGGCTGCTTTTAACTCTGGT-3'	5'-TCTCGCTCCTGGAAGATGGTG-3'
USP (*WIF-1*)	5'-GAATTTTATTGGTTGAAAGGGAGAT-3'	5'-AAAAATAAAAAAAACAAACAACACT-3'
MSP (*WIF-1*)	5'-AATTTTATTGGTTGAAAGGGAGAC-3'	5'-AAAAATAAAAAAAACAAACAACGCT-3'
BSP (*WIF-1*)	5'-GGAATTTTTAAATGTTGGGTGT-3'	5'-AAATAATAACTCCTATTCCTCCTCC-3'

WIF-1, WNT inhibitory factor 1; *DNMT*, DNA methyltransferase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; USP, unmethylation-specific primer; MSP, methylation-specific primer; BSP, bisulfate sequencing polymerase chain reaction.

CUGUUCCTT-3', which were constructed by GenePharma Co., Ltd. (Shanghai, China). A non-targeting siRNA was used as a negative control (NC-siRNA).

Transfection was performed with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol, when the NOZ cells were at ~90% confluence. The transfection efficiency was evaluated by counting the percentage of enhanced green fluorescent protein-positive NOZ cells using a fluorescence microscope. The cells were harvested 48 h following transfection to extract mRNA for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis and at 72 h to extract protein for western blot analysis.

RNA extraction and RT-qPCR analysis. Total RNA was extracted from the GBC cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then quantified using a spectrophotometer. cDNA was synthesized with the Revert Aid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) using 2 μg of RNA according to the manufacturer's protocol. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control. The primers for *WIF-1*, *c-Jun*, *DNMT1*, *DNMT3a*, *DNMT3b* and *GAPDH* were designed and synthesized according to standard primer design principles (Table I). PCR reactions were performed using FastStart Universal SYBR-Green Master Mix (Roche Diagnostics, Basel, Switzerland) on a 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following PCR conditions were used: Initial denaturation at 95˚C for 3 min, followed by 40 cycles at 95˚C for 15 sec and at 60˚C for 30 sec. Each sample was analyzed in triplicate. The relative expression levels of target genes were calculated based on normalization to the endogenous mRNA expression of *GAPDH*, prior to comparative analysis using the $2^{-\Delta\Delta Cq}$ method (21).

Protein extraction and western blot analysis. For protein extraction, the cells were lysed in RIPA lysis buffer containing a protease inhibitor mixture. The protein concentration was then measured using a bicinchoninic acid assay (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. A total of 20 μg of protein was subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare Life Sciences, Chalfont, UK). Following blocking with 5% nonfat milk for 3 h, the PVDF membranes were separately incubated with primary antibodies against c-Jun (1:1,000 dilution; cat. no. ab31419; Abcam), WIF-1 (1:1,000 dilution; cat. no. ab186845; Abcam), DNMT1 (1:1,000 dilution; cat. no. 5119; Cell Signaling Technology, Inc., Danvers, MA, USA), DNMT3a (1:1,000 dilution; cat. no. 3598; Cell Signaling Technology, Inc.), DNMT3b (1:1,000 dilution; cat. no. 67259; Cell Signaling Technology, Inc.) and β-actin (1:1,000 dilution; cat. no. sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4˚C. Following several washes with Tris-buffered saline with Tween (TBST), the appropriate secondary antibody conjugated to horseradish peroxidase (1:2,000 dilution; cat. no. ZB-2301 or ZB-2305; ZSGB-BIO, Beijing, China) was added to the membranes, followed by incubation for 1 h at room temperature. The quantities of each protein were visualized using ECL Advance reagent following use of a chemiluminescence western blot immunodetection kit (Invitrogen; Thermo Fisher Scientific, Inc.).

DNA extraction, methylation-specific PCR and bisulfate sequencing PCR (BSP). Genomic DNA was isolated from the cells and tissues using the TIANamp Genomic DNA kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's protocol. Subsequently, 2 μg of genomic DNA was converted with sodium bisulfite and subsequently cleaned using a commercial kit (EpiTect Bisulfite kit; Qiagen GmbH, Hilden, Germany).

EMBOSS CpGplot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/) (22) was use to predict a CpG island spanning nucleotides -562 to -308 following depositing the 2,000 bp prior to the transcriptional start site of the *WIF-1* gene promoter sequence (NC_000012), in which 19 CpG dinucleotides were present (Fig. 1A). The primers used to amplify bisulfite-treated genomic DNA (nucleotides -557 to -302) for BSP were designed using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) (23) and are listed in Table I. The amplified products were extracted
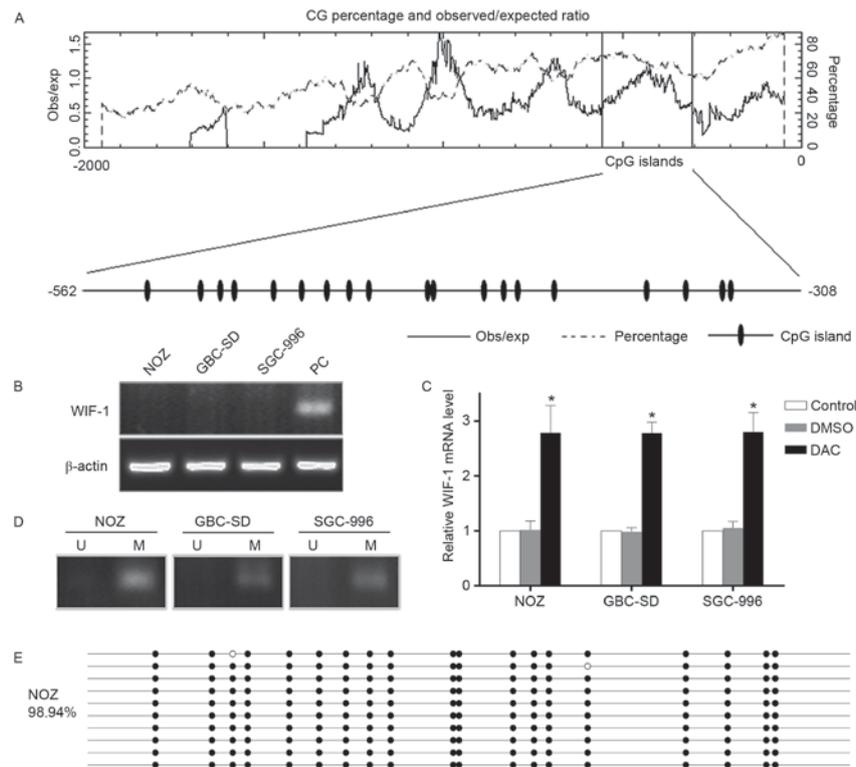
Figure 1. Loss of the expression of WIF-1 and aberrant hypermethylation of its promoter region in GBC cells. (A) A putative CpG island containing 19 CpG dinucleotides relative to the transcriptional start site meeting the following criteria: Percent C + percent G >50%; obs/exp ratio >0.60; length >200 bp. (B) Relative mRNA level of WIF-1 in GBC cell lines. β-actin served as a loading control. (C) mRNA expression of WIF-1 following DAC treatment in GBC cell lines. *P<0.05, vs. control group. (D) methylation-specific PCR assay of GBC cells. M bands are visible; U bands are not visible. (E) Bisulfite genomic sequencing of the WIF-1 promoter region in NOZ cells. Each circle corresponds to one CpG position; filled (black) circles correspond to methylated Cs; unfilled (white) circles correspond to unmethylated Cs. The percentage of methylated Cs was 98.94%. WIF-1, WNT inhibitory factor 1; GBC, gallbladder cancer; M, methylation-specific primer; U, unmethylation-specific primer; PC, positive control normal human lung tissue; obs, observed; exp, expected; DMSO, dimethyl sulfoxide; DAC, 5-aza-2'-deoxycytidine.

from a 1.5% agarose gel stained with ethidium bromide, using a gel extraction kit (E.Z.N.A.TM Gel extraction kit; Omega Bio-Tek, Inc., Norcross, GA, USA) and then purified for subcloning into the pMD18-T vector (Takara Bio, Inc., Otsu, Japan). Subsequently, 10 clones were randomly selected from each sample to determine the methylation status of the CpG islands of the WIF-1 promoter. The status of DNA methylation was determined using BiQ Analyzer software version 2.00 (http://biq-analyzer.bioinf.mpi-inf.mpg.de) (24).

For the methylation-specific PCR assay, bisulfite-treated genomic DNA was amplified using either an unmethylation-specific primer (USP) or a methylation-specific primer (MSP), as listed in Table I, targeting the WIF-1 promoter region sequence from -331/-330 to -164/-164, including five CpG islands. In this assay, results were defined as positive results when MSP bands were visible, with negative results defined as those with visible USP bands with or without MSP bands.

Co-immunoprecipitation assay. Soluble proteins were precleared with 1.0 µg of normal rabbit IgG and 20 µl of the resuspended volume of protein A/G plus-agarose (Santa Cruz Biotechnology, Inc.). Subsequently, 1 mg of total cellular protein was mixed with 3 µg of primary antibodies (c-Jun or DNMT1), following which 20 µl of the resuspended volume of protein A/G plus-agarose was added, and the samples were incubated at 4°C on a rotating device overnight. The

immunoprecipitated complexes were washed with lysis buffer and then analyzed via 10% SDS-PAGE and western blot analysis, using the respective specific antibodies.

Statistical analysis. The statistical analyses were performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA) or GraphPad Prism software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). Fisher exact test assessed the associations between the protein expression of WIF-1 and clinical pathological parameters. Pearson's coefficient was used for the calculation of correlations of the MOD values of WIF-1 and c-Jun, which represented the protein levels in GBC tissues. The measurement data are expressed as the mean ± standard deviation from at least three independent experiments and were analyzed using independent samples t-tests. Two-sided P<0.05 (two-sided) was considered to indicate a statistically significant difference.

Results

Loss of the expression of WIF-1 and aberrant hypermethylation of its promoter region in GBC cells. The results of the RT-qPCR analysis revealed the complete loss of WIF-1 in the GBC (NOZ, GBC-SD and SGC-996) cell lines analyzed (Fig. 1B). Treatment with the demethylating agent DAC effectively restored the mRNA levels of WIF-1 (Fig. 1C), suggesting that genome

Table II. Association between clinicopathological characteristics and the expression of *WIF-1* in gallbladder cancer.

Characteristic	Cases (n)	Expression of *WIF-1* -	+	P-value
Age (years)				0.723
<60	21	16	5	
≥60	29	24	5	
Sex				0.736
Male	23	19	4	
Female	27	21	6	
pT stage				0.171
T1-T2	20	14	6	
T3-T4	30	26	4	
Lymph node metastasis				0.171
Negative	20	14	6	
Positive	30	26	4	
Distant metastasis				0.138
Negative	34	25	9	
Positive	16	15	1	
Histological grade				0.707
High	16	12	4	
Moderate/poor	34	28	6	

WIF-1, WNT inhibitory factor 1.

methylation repressed the expression of *WIF-1* in these cells. To further verify the presence of aberrant hypermethylation, methylation-specific PCR analysis was performed on bisulfite-modified genomic DNA harvested from the GBC cells. A high level of amplification was observed with MSPs, compared with the absence of amplification with the USPs (Fig. 1D). BSP of the NOZ promoter region was subsequently performed. As shown in Fig. 1E, the percentage of methylated CpGs was as high as 98.94%. These findings indicated that the low expression level of *WIF-1* in the GBC cells was caused by aberrant hypermethylation of the promoter region.

Reduced expression of WIF-1 and correlation between protein levels of WIF-1 and c-Jun in GBC specimens. Negative or low expression levels of WIF-1 were observed in the cytoplasm and nucleus of the GBC mucosal cells using an immunohistochemistry technique. The expression of WIF-1 was only detected in 10 of the 50 gallbladder cancer samples, with a positive rate of 20%. By contrast, positive expression was detected in the majority of the cholecystitis tissue samples (18/20; 90%), which represented a significant difference (P<0.05). The associations between the expression of WIF-1 and clinicopathological characteristics of the GBC cases, including patient age and sex, pT stage, lymph node metastasis, distant metastasis and histological grade, are shown in Table II, which revealed that the expression of WIF-1 was not associated with any of these factors.

A high level of c-Jun-positivity was detected in serial sections of the same GBC specimens; a representative sample (case 4) is shown in Fig. 2A. By contrast, a high expression of WIF-1 and low expression of c-Jun was observed in case 26. There are currently no reports on the association between the expression of *WIF-1* and *c-Jun* in GBC specimens. To reveal correlations between these proteins, the present study used the MOD to semiquantitatively analyze the results. The data showed a negative correlation between the protein levels of WIF-1 and c-Jun in all GBC specimens (Pearson's r=-0.6213; P<0.05), as shown in Fig. 2B.

Knockdown of c-Jun increases the mRNA and protein expression of WIF-1. Among the cell lines examined (NOZ, GBC-SD and SGC-996 cells), the highest mRNA and protein expression levels of *c-Jun*, detected using RT-qPCR and western blot analyses, were found in the NOZ cells (Fig. 3A). Therefore, NOZ cells were used to further examine the role of *c-Jun* in GBC. To identify an effective siRNA for silencing the expression of *c-Jun*, three siRNA sequences (P-1, P-2 and P-3) were designed, chemically synthesized and transiently transfected into NOZ cells. The transfection efficiency was observed using fluorescence microscopy (blue light), which was high in all the groups listed above (Fig. 3B). The P-3/siRNA vector resulted in the most marked suppression of mRNA and protein expression of *c-Jun*, compared with the P-1/siRNA and P-2/siRNA vectors, whereas the NC/siRNA group had no effect (Fig. 3C). Therefore, for further experiments, P-3/siRNA was selected to induce knockdown, following which the mRNA and protein expression levels of *WIF-1* were examined. As shown in Fig. 3D, the expression of *WIF-1* was markedly restored in the P-3/siRNA group, which also supported the association between *c-Jun* and *WIF-1 in vitro*.

c-Jun represses the expression of WIF-1 by inducing hypermethylation of the WIF-1 promoter. The results of the BSP analysis of the *WIF-1* promoter in P-3/siRNA-transfected and DAC-treated NOZ cells were consistent with the expression data. Hypermethylation was observed for the majority of CpG dinucleotides within the amplified 255 bp region of the *WIF-1* promoter in NOZ cells (98.94%; Fig. 1E), however, a low level of CpG methylation was observed in the *c-Jun*/siRNA (79.47%) group (P<0.05; Fig. 4A). Of note, the patterns of DNA methylation in the *c-Jun*/siRNA NOZ cells were most similar to those in the DAC treatment group (74.73%; Fig. 4A), suggesting that *c-Jun* repressed the expression of *WIF-1* by inducing the hypermethylation of its promoter region.

c-Jun effects the methylation of WIF-1 through transcriptional regulation and interaction with DNMT1. Methyl groups are added to CG dinucleotides by DNMT3a/3b, and DNMT1 then maintains these DNA methylation patterns during DNA replication to ensure epigenetic gene silencing (8). In the present study, RT-qPCR and western blot analyses revealed that the knockdown of *c-Jun* in NOZ cells inhibited the expression of DNMT1, but had minimal effect on the expression of DNMT3a or DNMT3b (Fig. 4B).

In addition to the regulation of DNMTs, it was hypothesized that an alternative mechanism exists, whereby *c-Jun* can physically modulate epigenetically associated proteins
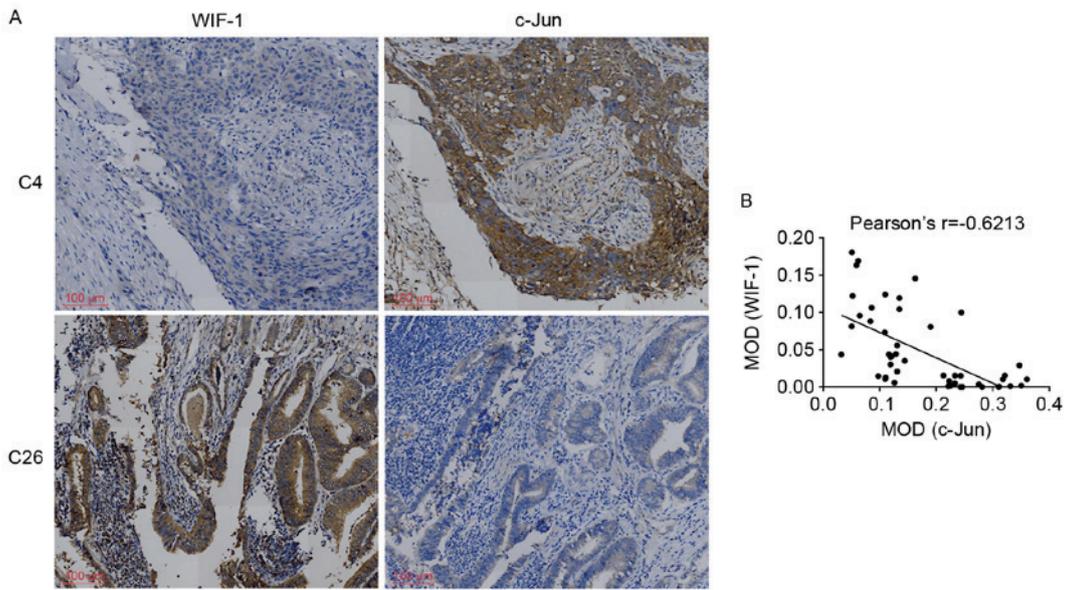
Figure 2. Correlation between protein levels of WIF-1 and c-Jun in GBC specimens. (A) Expression of WIF-1 and c-Jun were observed in the cytoplasm and nucleus of the GBC mucosa cells. C4, shows loss of expression of WIF-1 (left) with high expression of c-Jun (right). C26 shows high expression of WIF-1 (left) with low expression of c-Jun (right). (B) Protein levels of WIF-1 and c-Jun were negatively correlated in the 50 GBC specimens (Person's r=-0.6213; P<0.05). WIF-1, WNT inhibitory factor 1; GBC, gallbladder cancer; MOD, mean optical density; C4, case 4; C26, case 26.
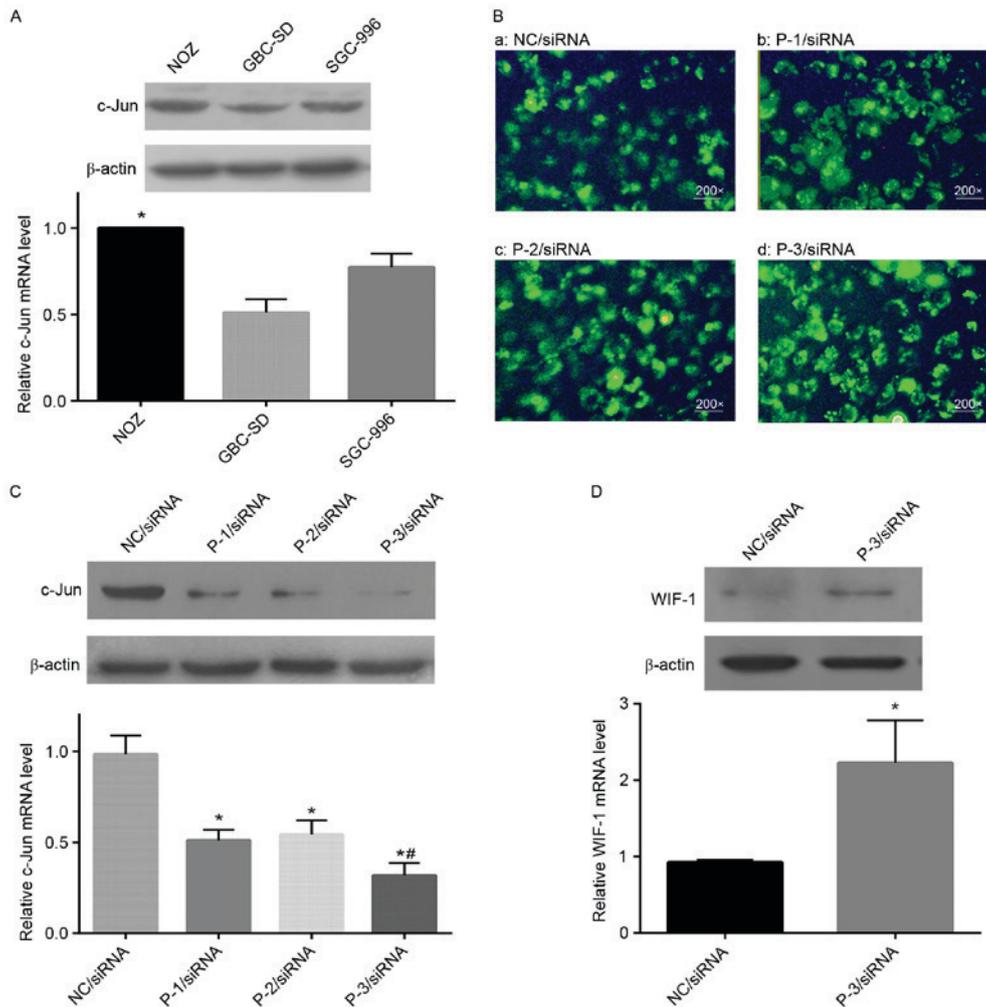

Figure 3. Knockdown of *c-Jun* affects the expression of *WIF-1*. (A) Relative mRNA and protein levels of *c-Jun* in GBC cell lines. *P<0.05, vs. GBC-SD and SGC-996 cells. (B) Transfection efficiency of *c-Jun* siRNA (NC/P-1/P-2/P-3) in NOZ cells. (C) Effects of *c-Jun*/siRNA transfection on the mRNA and protein expression of *c-Jun*. *P<0.05, vs. NC/siRNA group; #P<0.05, vs. P-1/siRNA and P-2/siRNA group. (D) Knockdown of *c-Jun* increased the mRNA and protein expression of *WIF-1*. *P<0.05, vs. NC/siRNA group. WIF-1, WNT inhibitory factor 1; GBC, gallbladder cancer; NC, negative control; siRNA, small interfering RNA.
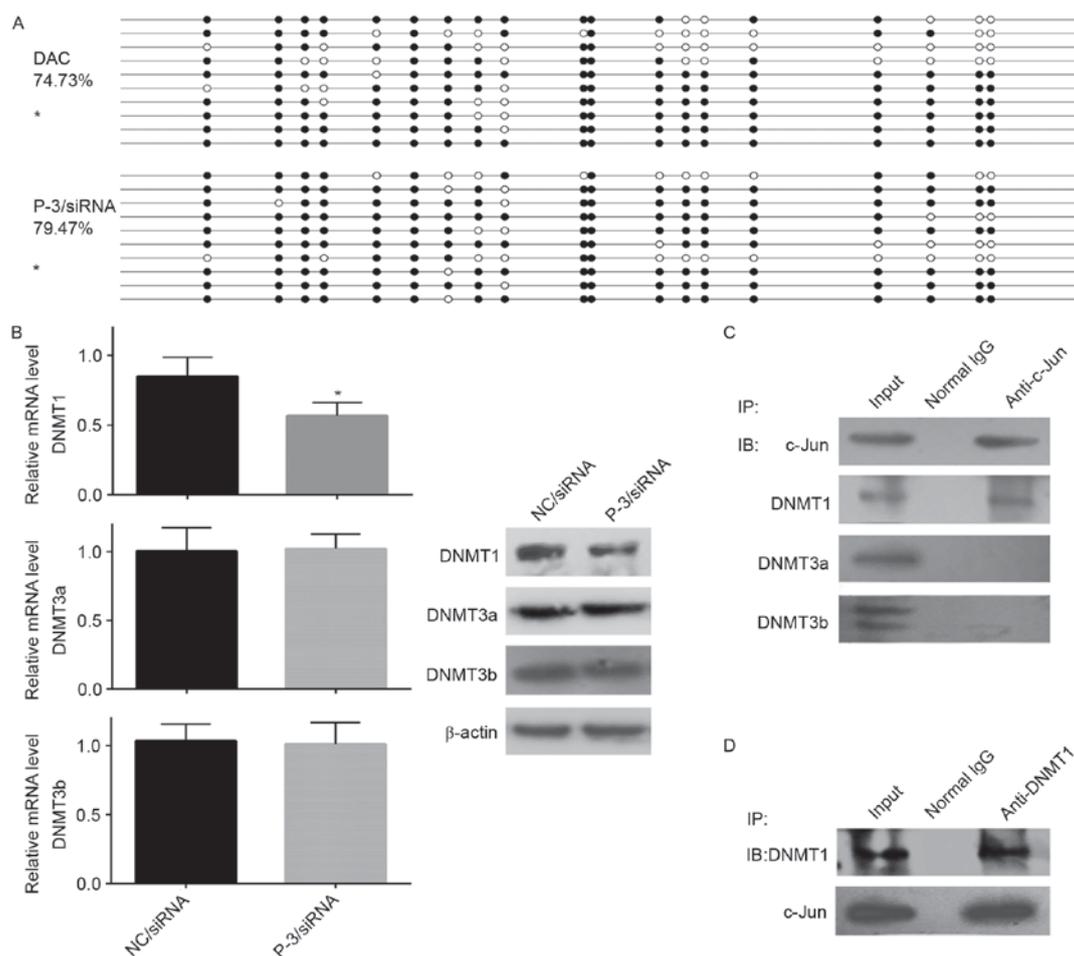
Figure 4. *c-Jun* affects *WIF-1* methylation through transcriptional regulation and interaction with DNMT1. (A) BSP of the *WIF-1* promoter region in P-3/siRNA-transfected and DAC-treated NOZ cells. Percentage of methylated Cs: DAC=74.73%, P-3/siRNA=79.47%. *P<0.05, vs. NOZ cells in Fig. 1E. (B) Knockdown of *c-Jun* decreased the mRNA and protein expression of DNMT1, but not that of DNMT3a and DNMT3b. *P<0.05, vs. NC/siRNA group. (C) Endogenous c-Jun immunoprecipitated DNMT1, but not DNMT3a or DNMT3b. (D) Reverse immunoprecipitation was performed using anti-DNMT1 antibody. Normal IgG served as a negative control. WIF-1, WNT inhibitory factor 1; DNMT; DNA methyltransferase; GBC, gallbladder cancer; NC, negative control; siRNA, small interfering RNA.

to trigger epigenetic modifications around the regulatory elements of *WIF-1*. Therefore, a co-immunoprecipitation assay was designed using NOZ cells. As shown in Fig. 4C, c-Jun was immunoprecipitated with DNMT1, but not DNMT3a or DNMT3b. Reverse immunoprecipitation with DNMT1 antibodies was then performed to confirm the interaction (Fig. 4D). These results indicated that the methylation of *WIF-1* associated with *c-Jun* was predominantly through DNMT1.

Discussion

WIF-1 is a key gene, which encodes a secreted protein that antagonizes the Wnt pathway and is involved in early embryonic development (25). As an important tumor suppressor, the abnormal expression of *WIF-1* can result in carcinogenesis through the dysregulation of cell proliferation and differentiation. It has been demonstrated that the expression of *WIF-1* is decreased in several types of tumor (10-13). The findings of the present study demonstrated a loss of the expression of *WIF-1* in GBC cell lines (NOZ, GBC-SD and SGC-996). The results also showed that treatment with DAC, a potent demethylating drug, which binds to DNA methylation

enzymes to inhibit their activity, restored the mRNA expression of *WIF-1*. In addition, the methylation-specific PCR and BSP assays showed that the hypermethylation of *WIF-1* DNA may explain its loss of expression in GBC cells.

Due to the relatively low incidence, there have been few investigations of the role of *WIF-1* in the pathogenesis of GBC. In the present study, the protein expression of WIF-1 was examined in 50 GBC and 20 cholecystitis specimens using imunohistochemical techniques. A reduced expression of WIF-1 was detected in the GBC specimens, which indicated that *WIF-1* was important in the pathogenesis of GBC. However, in contrast to previous reports of other tumors, subgroup analysis showed no correlation between the expression of WIF-1 and key clinicopathological parameters in GBC, including age and sex, pT stage, lymph node metastasis, distant metastasis and histological grade. This suggested that *WIF-1* may be involved in the tumorigenesis of GBC only, and not the progression.

There have been few investigations to date regarding the upstream molecular mechanism for regulating *WIF-1*. In the present study, it was observed that the protein levels of WIF-1 and c-Jun were negatively correlated in serial

sections of 50 GBC specimens, suggesting an association between *c-Jun* and *WIF-1*. RNA interference was used to for the *in vitro* knockdown *c-Jun*, and an effective siRNA was obtained (P-3/siRNA). The mRNA and protein expression levels of *WIF-1* were then detected in NOZ cells transiently transfected with P-3/siRNA, and the results demonstrated that silencing of the *c-Jun* gene upregulated the expression of *WIF-1*. Considering this evidence, it was hypothesized that *c-Jun* may be involved in regulating the expression of *WIF-1* in GBC.

The results described above prompted further examination of the specific molecular mechanism underlying the *c-Jun*-induced downregulated expression of *WIF-1*. The epigenetic modification of cellular genes via aberrant methylation in promoter regions has been identified as a crucial mechanism for inactivating tumor suppressor genes in tumors (4,5,26). The present study showed silencing of *WIF-1* through hypermethylation of its promoter region in GBC cells. With these previous findings in consideration, it was hypothesized that *c-Jun* repressed the expression of *WIF-1* by modifying the promoter methylation status of the *WIF-1* gene. To confirm the factors involved, BSP assays were performed, and the obtained data showed that CpG islands in the *WIF-1* promoter region were hypermethylated at a higher frequency in NOZ cells, compared with those in the knockdown group (P-3/siRNA cells), revealing a similar methylation ratio to that observed in the DAC-treated group. These results suggested that the knockdown of *c-Jun* caused the demethylation of CpG islands in the *WIF-1* promoter region. Taken together, these findings are confirmed the hypothesis that *c-Jun* can repress the expression of *WIF-1* through epigenetic modification by inducing hypermethylation of the *WIF-1* promoter in GBC.

Methylation modifications are mediated by DNMTs through various mechanisms. DNMT1 and DNMT3b have been shown to be responsible for the aberrant hypermethylation of *WIF-1* in the HCT116 colon cancer cell line (10). The present study investigated whether *c-Jun* affected DNMTs in GBC cells. The experimental results demonstrated that *c-Jun* knockdown in the NOZ cells inhibited the expression of DNMT1, but not that of DNMT3a or DNMT3b. A co-immunoprecipitation assay was performed to examine the mechanism involved in the chromatin remodeling induced by *c-Jun*, and it was found that c-Jun interacted with DNMT1 only, and not with DNMT3a or DNMT3b. Taken together, these results showed that *c-Jun* affects the methylation of *WIF-1* through transcriptional regulation and interaction with DNMT1, a critical DNA methyltransferase.

However, the results of the present study were based on data obtained using GBC cells and partial GBC tissues. Unlike the findings in the present study, a previous study reported that *WIF-1* was expressed in the majority of colorectal cancer specimens (27). The downregulation of *c-Jun* has been observed in specific types of human cancer, including breast cancer; these discrepancies may be due to the different types and developmental stages of the cells examined (28,29). Therefore, the regulatory mechanisms of *c-Jun* and *WIF-1* are complicated, however, the results of the present study assist in elucidating the mechanisms linking these proteins in GBC.

In conclusion, the present study found that the expression of *WIF-1* was low in GBC cells due to aberrant hypermethylation of its promoter region. Additionally, an alternative pathogenesis of GBC was indicated in which *c-Jun* causes hypermethylation of the *WIF-1* promoter region, and represses the expression of *WIF-1* through transcriptional regulation and interaction with DNMT1 as an early event in the tumorigenesis of GBC.

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