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Zinc finger protein 278, a potential oncogene in human colorectal cancer

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Zinc finger protein 278 (ZNF278) is a novel Krueppel Cys2-His2-type zinc finger protein that is ubiquitously distributed in human tissues. Whether ZNF278 is related to the development of colorectal cancer is still unclear. The transcriptional level of *ZNF278* was studied in colorectal cancer by real-time polymerase chain reaction. The results showed that ZNF278 expression was increased in 53% of colorectal cancer tissues compared to corresponding non-cancerous tissues. The transcriptional down-regulation of *ZNF278* was detected in only three (6%) human colorectal cancer tissues compared to corresponding non-cancer tissues. No significant difference was detected in 19 (41%) pairs of samples. However, we failed to find a significant association between the up-regulation of *ZNF278* transcription and age, sex, the degree of infiltration, or the tumor size of colorectal cancer. To study the function of ZNF278 in colorectal carcinogenesis, the colon cancer cell line SW1116 was stably transfected with a wild-type ZNF278 plasmid to construct an overexpression system, and was transiently transfected with the small interfering RNA of ZNF278 to construct a ZNF278 knockdown system. Cell proliferation was assessed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide dye and a cell counter. The results show that ZNF278 promotes cell growth, and its knockdown suppresses cell proliferation. ZNF278 could be a potential proto-oncogene in colorectal cancer.

Keywords ZNF278; proto-oncogene; colorectal cancer; cell cycle; cell proliferation

Colorectal cancer has traditionally been one of the most common malignancies in Europe and North America, whereas cancers of the upper gastrointestinal tract and liver have predominated in Asian populations [1]. However, during the past few decades, there has been a remarkable rise in the incidence of colorectal cancer in Asia [2]. Colorectal carcinomas arise through aberrant expression of some oncogenes and tumor suppressor genes [3,4]. Identification of novel cancer-related genes will contribute to the understanding of the mechanism of colorectal carcinogenesis.

The zinc finger protein gene family is large, and 1% of all human genes could belong to this superfamily [5]. Among this family, the Cys2-His2(C2H2) subtype is the largest subfamily, including approximately 700 proteins [6]. Zinc finger proteins have important physiological functions in cell proliferation and differentiation [7–10]. Their aberrant expression is related to various diseases, including cancers [11–15].

Zinc finger protein 278 (ZNF278), also named POZ/BTB and AT-hook-containing zinc finger protein (PATZ), is a recently identified transcription factor with seven C2H2-type zinc fingers [16]. ZNF278 belongs to the Krueppel C2H2-type zinc finger protein family [16]. It is a novel zinc finger protein that is ubiquitously distributed in human tissues. Although the physiological role of ZNF278 is not clear, experimental evidence suggests that it is a potential transcription repressor [16,17]. In small round cell sarcoma, this gene is fused to Ewing sarcoma (EWS) gene by a small inversion in 22q; the hybrid is then thought to be translocated t(1;22)(p36.1;q12). The rearrangement of chromosome 22 involves intron 8 of EWS and exon 1 of this gene, thus creating a chimeric sequence containing the transactivation domain of EWS fused to the zinc finger domain of this protein [18]. However, whether ZNF278 is related to other primary cancers is still unknown.

The aim of this study was to detect the expression level of *ZNF278* in human colorectal cancer samples, and study the basic function of *ZNF278* in a human colorectal cancer cell line.

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Materials and Methods

Tissue samples and cell line

Forty-seven colorectal cancer tissues were obtained from patients undergoing surgery before chemotherapy at Renji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, in compliance with our Institutional Review Board. From each patient, we obtained adjacent tumor-free parenchyma from a region located 5 cm from the tumor to serve as a paired control. Immediately after surgical removal, tissue samples were snap-frozen in liquid nitrogen then maintained at −80 ºC until use. Colon cancer cell line SW1116 cells were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (Gibco BRL) under 5% CO_2 humidified atmosphere and at 37 ºC as previously described [19].

Real-time RT-PCR for ZNF278 mRNA expression

Total RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen/Gibco BRL, Carlsbad, USA). RT reactions were carried out using 5 μg total RNA in a final reaction volume of 20 μl and Superscript II reverse transcriptase (Invitrogen). Relative quantitation data were obtained using the comparative Ct method with the ABI PRISM 7700 Sequence Detection System (software version 1.6; ABI, Foster City, USA) according to the manufacturer's protocol. The primers for ZNF278 were: F, 5′-GCAGACACAGCACGGAGAT-3′; and R, 5′-CGCTGAACACCGACTCAAAGT-3′. Real-time PCR was also carried out using the primers for β-actin to normalize each of the extracts for amplifiable human RNA. The results were expressed as the ratio of copies of target genes to β-actin. The Ct values were measured, and the average Ct of the triplicate samples was calculated. Significant alteration in mRNA expression was defined as a 3-fold difference in the expression level between cancer tissues and adjacent non-cancerous tissues.

Construction of expression vectors and stable transfection

To construct the wild-type ZNF278 (GenBank accession No. NM_032050) expression vector, a PCR-generated full-length ZNF278 cDNA was inserted into the *Eco*RI-*Hin*dIII sites of the expression vector pcDNA3.1/Myc-histone A (kindly gifted by Dr. Xiaoqing Chen, Shanghai Jiaotong University, Shanghai, China). The plasmid, pcDNA3.1-ZNF278, was confirmed by DNA sequence analysis. Nested PCR was carried out to amplify the full-length ZNF278 cDNA. The following primers were used: F1, 5′-CGGCGCACCTGCGAGACTACAGA-3′ and R1, 5′-TCC-CAGCAGTCCCCAGATGGTTGT-3′ for the first PCR; and F2, 5′-CCCAAGCTTCCATGGAGCGGGTGAAC-3′ and R2, 5′-CCGGAATTCTTTCCCTTCAGGCCCCAT-3′ for the second PCR. Before transfection, 5×10^5 SW1116 cells were seeded in 6 cm wells. The cells were transfected with 1 μg of either pcDNA3.1-ZNF278 or pcDNA3.1 using Effectene Transfection Reagent (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. After 24 h, the medium was replaced with a fresh medium. The cells were further incubated for 24 h, and the medium was replaced with that containing 300 μg/ml G418 for approximately 30 d, and the medium was replaced every day. Overexpression of ZNF278 was confirmed by real-time RT-PCR and Western blot analysis using anti-c-Myc tag sequence antibody (Sigma, St. Louis, USA).

RNA interference and transient transfections

ZNF278 small interfering RNA (siRNA) (sense, 5′-GCGCCGAUAUAAUGCUCUUTT-3′ and antisense, 5′-AAGAGCAUUAUAUCGGCGCGG-3′) and negative control siRNA (sense, 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense, 5′-ACGUGACACGUUCGGAGAATT-3′) were designed and synthesized (Shanghai GenePharma, Shanghai, China). Mock-transfected or pcDNA3.1-ZNF278-transfected SW1116 cells were transfected using 80 nM of each siRNA duplex. The siRNA was complexed with the transfection reagent in a serum- and antibiotic-free medium for 8 h. After applying the transfection reagents, the cellular medium was replaced with the serum-containing maintenance medium, and the cells were incubated for 48 h. Selective silencing of ZNF278 was confirmed by real-time RT-PCR and Western blot analysis using anti-c-Myc tag sequence antibody (Sigma).

Cell viability assay

Cell growth was assessed using 3-(4,5-dimethylthiazol-2-l)-2,5-diphenyltetrazolium bromide (Sigma) with an absorption maxima at 570 nm, according to the manufacturer's instructions. Briefly, 5×10^3 cells stably transfected with pcDNA3.1-ZNF278 or pcDNA3.1 were seeded per well in a 96-well flat-bottom plate. The cells were allowed to grow for 48 h, and 20 μl 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (5 mg/ml in phosphate-buffered saline) was then added to each well. After 3 h incubation at 37 ºC, the cells were lysed by the addition of 0.1 M HCl in isopropanol alcohol; this produced a color, the absorbance of which was monitored at 570 nm. In addition, 5×10^3 SW1116 cells were

transfected in complete medium containing 80 nM of siRNA-ZNF278 or siRNA-negative control for 48 h, and cell viability was then assayed.

Cell proliferation studies

Cells in the log growth phase (7×10^4) stably transfected with either pcDNA3.1-ZNF278 or pcDNA3.1 plasmid were seeded in a 24-well flat-bottom plate for the assessment of *in vitro* cell growth. These cells were trypsinized and counted on a Casy Counter (Schaerfe System, Reutlingen, Germany) for 6 d. The SW1116 cells (5×10^4) were transfected with 80 nM siRNA-ZNF278 or siRNA-negative control, and the cell number was counted at 48 h after transfection.

Flow cytometry for the detection of cell cycle progression

Cell cycle analysis was carried out by flow cytometry. Approximately 1×10^6 cells were removed and washed twice with phosphate-buffered saline and fixed in ice-cold ethanol for 1 h. The samples were then concentrated by removal of ethanol and exposure to 1% (*V*/*V*) Triton X-100 (Sigma) and 0.01% RNase (Sigma) for 10 min at 37 ºC. Cellular DNA was stained in the dark with 0.05% propidium iodide for 20 min at 4 ºC. Cell cycle distributions were determined using a flow cytometer (FACSCalibur; Becton Dickinson, San Jose, USA). The data obtained from 10,000 cells were analyzed using the MultiCycle software package (Phoenix Flow Systems, San Diego, USA).

Statistical analysis

Data are representative of at least three independent experiments carried out in triplicate, and are presented as the mean±SD. Comparisons between groups were made using Student's *t*-test. The data for tissue sample groups were compared using the Sign test. Relationships were analyzed by Fisher's exact test using SAS 6.12 software (SAS Institute, Cary, USA). A value of $P<0.05$ was taken to indicate a significant difference between the mean values.

Results

ZNF278 expression is up-regulated in human colorectal cancer tissue

Real-time quantitative PCR was carried out to evaluate the amounts of *ZNF278* mRNA in colorectal cancer samples (n=47) and the corresponding non-cancerous samples (n=47). ZNF278 transcription was found to be significantly up-regulated in 25 (53%) human colorectal cancer tissues compared to the corresponding non-cancer tissues (χ^2=15.75, $P<0.05$) (**Fig. 1**). The transcriptional down-regulation of *ZNF278* was detected in three

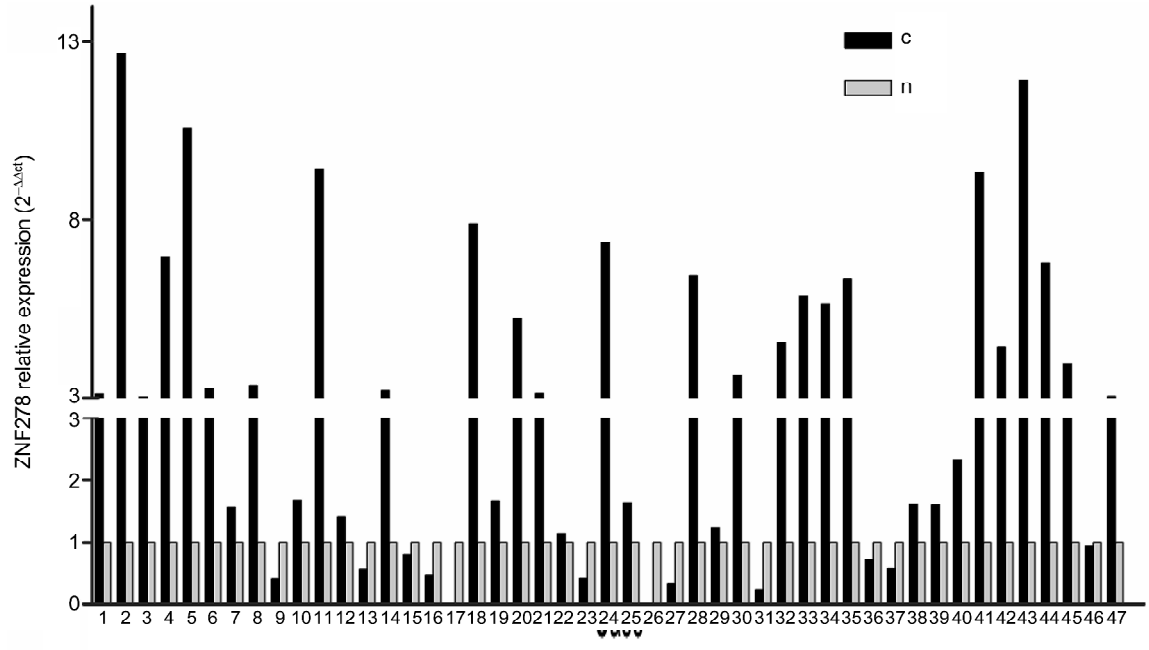

Fig. 1 Relative expression of zinc finger protein 278 (ZNF278) in primary colorectal carcinomas Transcriptional up-regulation of ZNF278 was detected in 25 colorectal cancer tissues compared to non-cancerous tissues (χ^2=15.75; $P<0.05$) from the same patients. c, colorectal cancer tissue; n, corresponding non-cancerous tissue.

(6%) human colorectal cancer tissues compared to the corresponding non-cancerous tissues (**Fig. 1**). No significant difference was detected in 19 (41%) colorectal cancer tissues and the corresponding non-cancerous tissues (**Fig. 1**). There were no significant association in the up-regulation of ZNF278 expression and age, sex, the degree of infiltration, or tumor size of colorectal cancer (data not shown).

ZNF278 functions as a potential proto-oncogene in colorectal cancer

To assess the function of ZNF278 in colorectal cancer, we cloned human ZNF278 cDNA in the expression vector pcDNA3.1/Myc-histone A. The siRNA was transiently transfected in order to knock down the ZNF278 expression. Overexpression of ZNF278 in the stable selected transfectants and knockdown of ZNF278 by siRNA transfection were confirmed by real-time RT-PCR and Western blot analysis (**Fig. 2**). We first examined the proliferative effects of ZNF278 on the colorectal cancer cells. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assays showed that the absorbance of the pcDNA3.1-ZNF278-transfected cells was 0.84±0.06, and that of the pcDNA3.1-transfected cells was 0.58±0.03. The absorbance of the siRNA-ZNF278-transfected cells was 0.67±0.03, whereas that of siRNA-negative control-transfected cells was 0.88±0.02. Student's *t*-test indicated a significant difference between the groups ($P<0.01$). In addition, the cell growth curve showed that the *in vitro* tumor cell growth was significantly promoted in the cells

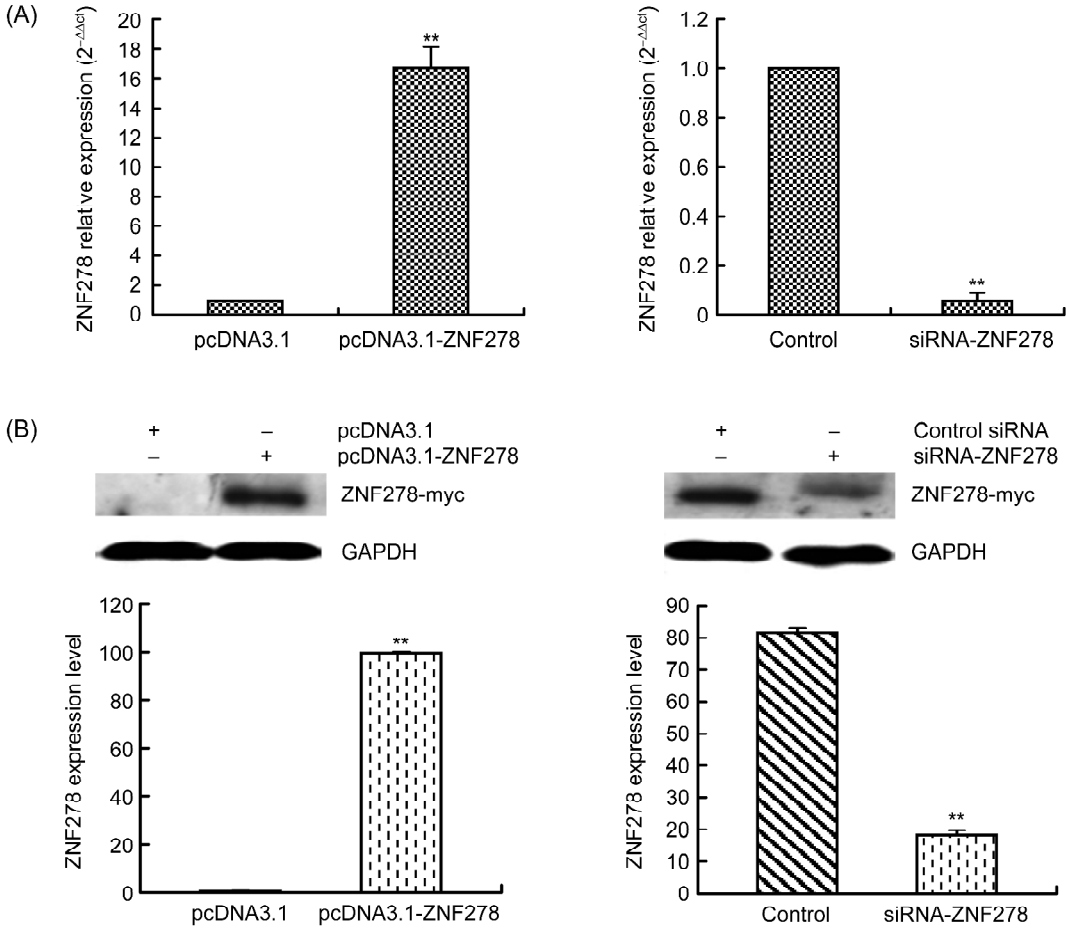

Fig. 2 Overexpression and knockdown of zinc finger protein 278 (ZNF278) in SW1116 colon cancer cells SW1116 cells were stably transfected with pcDNA3.1 or pcDNA3.1-ZNF278 to identify the overexpression system. Cells stably transfected with pcDNA3.1-ZNF278 were transiently transfected with the indicated small interfering RNAs (siRNAs) to identify the knockdown of ZNF278. (A) The overexpression and knockdown effects were assessed by real-time RT-PCR. (B) The overexpression and knockdown effects were assayed by Western blot analysis using anti-c-myc antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for the loading control. Quantification of ZNF278-myc protein bands relative to GAPDH is shown below the blots. +, transfection; –, non-transfection. $^{**}P<0.01$.

transfected with the ZNF278 plasmid as compared to control cells [**Fig. 3(A)**, $P<0.01$]. Cell counting assay showed that the numbers of the cells transfected with siRNA-ZNF278 and siRNA-negative control were (4.66±0.35)×10^5 and (5.50±0.53)×10^5, respectively. Knockdown of ZNF278 significantly inhibited cell growth [**Fig. 3(B)**, $P<0.01$]. Our results indicated that transfection of the SW1116 cells with pcDNA3.1-ZNF278 promoted cell growth, and siRNA transfection resulted in a significant inhibition of cell growth ($P<0.01$).

To further study the function of ZNF278 on the cell cycle, we evaluated the cell cycle of the cells stably transfected with pcDNA3.1-ZNF278 or pcDNA3.1 and of the cells transiently transfected with siRNA-ZNF278 or siRNA-negative control. As shown in **Table 1**, the overexpression of ZNF278 in the cells transfected with the pcDNA3.1-ZNF278 plasmid significantly increased the percentage of the S-phase cells and decreased the percentage of the G_0/G_1-phase cells ($P<0.05$) [**Fig. 4(A,B)**]. The knockdown of ZNF278 expression significantly blocked the cell cycle at the G_0/G_1 phase ($P<0.05$) [**Fig. 4 (C,D)**].

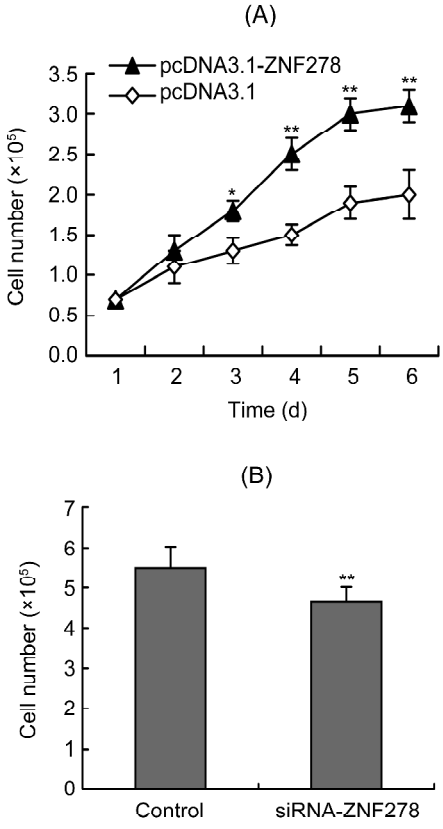

Fig. 3 Effect of zinc finger protein 278 (ZNF278) on proliferation of SW1116 colon cancer cells (A) Cell growth curves of stable transfection. Stable transfection of pcDNA3.1-ZNF278 significantly promoted cell growth, compared to the control. pcDNA3.1, SW1116 cells stably transfected with the pcDNA3.1 plasmid; pcDNA3.1-ZNF278, SW1116 cells stably transfected with the pcDNA3.1-ZNF278 plasmid. (B) Effect of small interfering RNA (siRNA)-ZNF278 on the growth of SW1116 cells. Transient transfection with siRNA-ZNF278 significantly inhibited cell proliferation. Control, SW1116 cells transfected with siRNA-negative control; si-ZNF278, SW1116 cells transfected with siRNA-ZNF278. *$P<0.05$; ** $P<0.01$.

Table 1 Influence of transfection on the cell cycle of SW1116 colon cancer cells (mean±SD) (%)

Groups	G_0/G_1	S	G_2/M
pcDNA3.1	76.57±0.71	21.80±0.10	1.67±0.67
pcDNA3.1-ZNF278	40.40±1.84*	36.87±0.12*	22.73±1.88*
siRNA-negative control	46.73±1.18	30.77±2.97	22.47±2.58
siRNA-ZNF278	52.50±3.28*	24.87±1.90*	22.63±3.16

Results from three independent experiments. pcDNA3.1, SW1116 cells stably transfected with the pcDNA3.1 plasmid; pcDNA3.1-ZNF278, SW1116 cells stably transfected with the pCDNA3.1-zinc finger protein 278 (ZNF278) plasmid; siRNA-negative control, SW1116 cells transfected with small interfering RNA (siRNA)-negative control; siRNA-ZNF278, SW1116 cells transfected with siRNA-ZNF278. *$P<0.05$.

Discussion

Colorectal cancer is a common malignant tumor worldwide, with the incidence increasing in Asian countries [2]. Aberrant gene expression is involved in colorectal carcinogenesis [3,4].

The zinc finger domain is a typical feature of zinc finger proteins. It consists of several cysteine and histidine residues, and the fold is created by the binding of specific amino acids in the protein to a zinc atom [20]. Many zinc finger proteins belong to the C2H2-type zinc finger protein family [21,22]. Zinc finger protein possibly targets the gene promoter region and regulates gene expression [23,24]. Zinc finger proteins have important physiological functions in human development and differentiation. For example, Egr-1 controls cell proliferation and apoptosis [25]. Aberrant expression of zinc finger proteins is related to various diseases, including cancers [11–15]. For example, aberrant expression of *KLF6* and *ST18* is associated with hepatocellular carcinoma and breast cancer, respectively [12,14]. ZNF278 is a novel zinc finger protein and might function as a transcription repressor [16, 17]. Therefore, whether it is involved in carcinogenesis is an interesting topic for study.

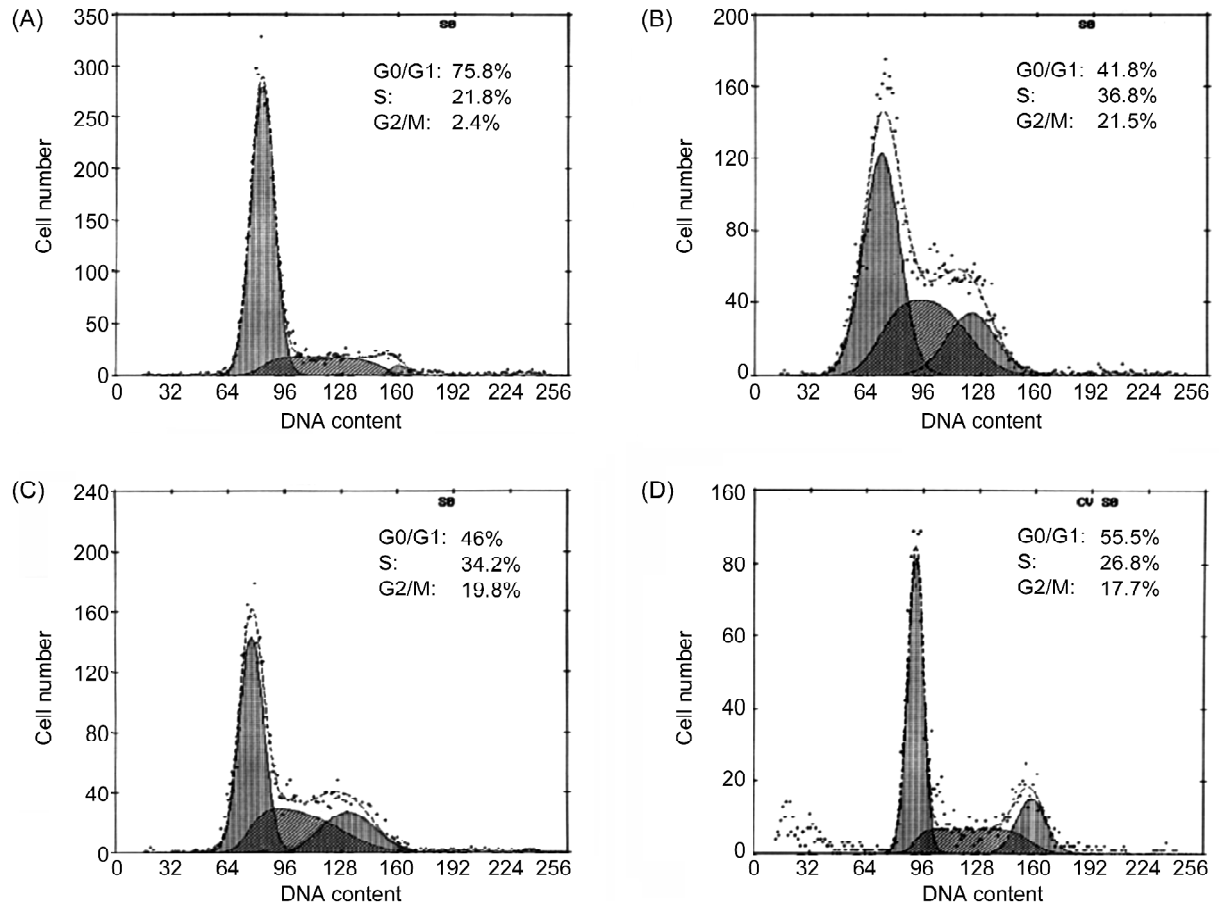

Fig. 4 Flow cytometry analysis of SW1116 colon cancer cells The overexpression of zinc finger protein 278 (ZNF278) increased the percentage of S-phase cells and decreased the percentage of G_0/G_1-phase cells. The knockdown of ZNF278 blocked the cell cycle at the G_0/G_1 phase. Cells were treated with stable transfection with pcDNA3.1 (A), stable transfection with pcDNA3.1-ZNF278 (B), transient transfection with 80 nM small interfering RNA (siRNA)-negative control after 48 h (C), and transient transfection with 80 nM siRNA-ZNF278 after 48 h (D).

The ZNF278 protein contains an AT-hook DNA-binding motif that usually binds to other DNA-binding structures to play an important role in chromatin modeling and transcription regulation [16,17]. Its Poz domain is thought to function as a site for protein-protein interaction and is required for transcriptional repression [16,17]. ZNF278 belongs to the C2H2-type zinc finger protein family. Some studies have supported that C2H2-type zinc finger proteins regulate cell proliferation, growth, differentiation, and carcinogenesis [9,10,26,27]. The ZNF278 protein has typical features of a transcription factor. It was suggested to be a transcription repressor [16,17]. Based on this research, ZNF278 might be considered to be an important factor in the physiological state. Aberrant expression of ZNF278 could lead to disease. In one published report, the rearrangement of the *ZNF278* gene was detected in small round cell sarcoma [18]. However, it is still unknown whether *ZNF278* is related to other primary cancers.

In the present study, we examined the ZNF278 expression level in colorectal cancer tissues and corresponding non-cancerous tissues, and found that the ZNF278 expression was significantly higher in cancer tissues than in the non-cancerous tissues. This suggested that the up-regulation of ZNF278 expression might contribute to colorectal tumor carcinogenesis. In particular, ZNF278 is a type of zinc finger protein and contains domains involved in DNA-binding and protein-protein interactions. It is possible that ZNF278 is involved in some important signaling pathways or regulates the transcription of other important genes. However, we failed to find an association between the ZNF278 expression level and age, sex,

the degree of infiltration, or tumor size of colorectal cancer. Possibly, ZNF278 influences the initiation but not the progression of colorectal cancer. This inference has to be verified in the future. In addition, because of absence of specific antibodies against ZNF278, protein expression levels under biological state could not be analyzed by Western blot or immunohistochemical methods. It will be useful to study the expression of ZNF278 protein in tumor tissues in the future.

In order to identify the function of ZNF278, we constructed a wild-type ZNF278 expression vector and transfected the SW1116 cells. In addition, we transiently transfected the SW1116 cells with ZNF278 siRNA. We studied the effect of ZNF278 on the biological function of the cells with regard to the overexpression and knockdown of ZNF278. The results of our study revealed that ZNF278 promoted colorectal cancer cell growth, and that the knockdown of ZNF278 suppressed cell growth and arrested the cell cycle. According to the abovementioned results, the function of ZNF278 is similar to that of other proto-oncogenes such as *c-myc* [28]. *ZNF278* could be a potential proto-oncogene in colorectal carcinoma. Unfortunately, the knockdown of ZNF278 did not induce apoptosis (data not shown). It is likely that ZNF278 does not influence the signal pathway of apoptosis.

In summary, the up-regulation of *ZNF278* expression was observed in human colorectal cancer tissues. ZNF278 might be a potential oncogene in colorectal cancer.

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