Value of *CNRIP1* promoter methylation in colorectal cancer screening and prognosis assessment and its influence on the activity of cancer cells

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Abstract

Introduction: The aim of the study was to investigate the effect of *CNRIP1* promoter methylation on the proliferative, invasive and migration potential of colorectal cancer cells, including its potential use for the early detection and prognostic assessment of colorectal cancer.

Material and methods: Quantitative methylation-specific PCR (qMSP) was used to detect the methylation status of the *CNRIP1* promoter region in peripheral blood samples drawn from patients with colorectal adenocarcinoma, benign colorectal adenoma, and matched healthy controls. Putative CpG methylation sites were then pyrosequenced. We subsequently suppressed *CNRIP1* methylation within colon cancer cells via treatment with 5-azacytidine and overexpressed colon cancer cells by transfection with a *CNRIP1*-overexpression pcDNA3.0 plasmid. Thereafter, the *CNRIP1* methylation status and mRNA and protein expressions levels were determined. Finally, the proliferative, invasive and migration abilities of cell lines were determined with the CCK-8 and Transwell cell assays.

Results: There were differences in the methylation status at loci 2216, 2226, 2231, 2245, and 2254 within the promoter region of *CNRIP1* between patients with colorectal adenocarcinoma, colorectal adenoma, and healthy volunteers. The methylation status of CpG sequence 2245 significantly correlated with tumor diameter, invasion depth, TNM stage, grade, and lymph node metastasis ($p < 0.05$). The proliferative, invasive and migration abilities of colon cancer cells treated with 5-azaC or transfected with a *CNRIP1*-overexpression plasmid were significantly impaired relative to negative controls ($p < 0.05$).

Conclusions: The methylation status at locus 2245 within the CNRIP1 promoter region has potential value for the early detection and prognostic evaluation of colorectal cancers. Demethylation of the *CNRIP1* promoter or overexpression of *CNRIP1* can reduce the proliferative and migration abilities of colon cancer cells.

Key words: colorectal adenocarcinoma, cannabinoid receptor interacting protein 1 (*CNRIP1*), early detection, methylation, prognostic evaluation, screening, tumor cell activity.

Introduction

Colorectal cancer (CRC) is a formidable burden worldwide. Globally, there are 100 million new CRC cases per annum, with ~21% of those proving fatal [1]. Whilst outcomes are generally favorable if detected and treated early, when 74% of patients can expect to live 5 years, 25% of all CRCs are only detected when the tumor has metastasized, when only 6% of patients survive 5 years. Approximately 50% of patients die from early or postoperative metastasis or cancer recurrence [2–4].

An estimated 93% of CRCs evolve from a premalignant adenoma via the so-called adenoma-carcinoma sequence [3]. This transitory period is lengthy (5–7 years [2]), which provides a window of opportunity where dysplastic lesions can be detected and excised at the precancerous phase, which is generally curative, or at an early, localized cancerous stage, which is highly treatable and associated with favorable outcomes. The discovery of molecular markers specific to precancerous colorectal adenomas and early, localized colorectal cancers would surely aid their early detection and prognosis assessment.

Hypermethylation of DNA sequences within promoter regions of crucial genes is an early event of tumor development, occurring even before cancer gene mutations [5, 6]. DNA methylation is a method of gene silencing in both prokaryotes and eukaryotes, whereby the enzyme DNA methyltransferase adds a methyl group at the fifth carbon of cytosines immediately followed by a guanine. Blood samples are favored for surgical or endoscopic biopsies as screening specimens, and aberrant DNA methylation profiles are more stable in blood samples compared to circulating mRNA [7–10]. Accordingly, aberrant DNA methylation patterns may serve as a screening tool for CRC [7, 11–14].

Hypermethylation of the promoter region of cannabinoid receptor interacting protein 1 (*CNRIP1*) has been observed in colorectal adenocarcinoma tissue [15]. Current evidence suggests that CNRIP1 regulates the eye and neural development of *Xenopus laevis* [16]. Furthermore, aberrant DNA methylation biomarkers have been used to detect cholangiocarcinoma in biliary brush samples [17] and diffuse-type early gastric carcinogenesis [18]. However, the correlation between the methylation status of the *CNRIP1* promoter region within peripheral blood of patients with colorectal adenocarcinoma and its association with clinicopathological characteristics has not yet been reported. Accordingly, the present study sought to evaluate the use of *CNRIP1* methylation for the early detection and prognostic assessment of colorectal cancers. We used quantitative methylation-specific polymerase chain reaction (PCR) (qMSP) technology [19] to detect the methylation levels of the CpG island in the *CNRIP1* promoter region in peripheral blood drawn from patients diagnosed with colorectal adenocarcinoma and colorectal adenoma and within the peripheral blood samples drawn from healthy volunteers. Furthermore, the proliferative, invasive and migration potential of colon cancer cells induced to have either suppressed methylation or *CNRIP1* overexpression were determined in parallel to that of unaltered colon cancer and normal colon cell lines.

Material and methods

Ethical review

The present study was ethically approved by the Clinical Ethics Committee and Research Ethics Committees at the First Affiliated Hospital of Huzhou University, China.

Blood samples

Blood samples were collected from 100 CRC patients enrolled in our hospital from March 2013 to January 2014 (28 males, 22 females; 48–82 years old, 69.35 on average), 20 colorectal adenoma patients (10 males, 10 females; 38–61 years old, 52.61 on average), and 20 healthy volunteers (10 males, 10 females; 37–75 years old, 49.23 on average). Patients with colorectal adenocarcinoma or adenoma were admitted to hospital for the first time and had no cancer treatment before the blood draw. Patients with the following diagnoses were excluded: familial adenomatous polyposis (FAP), hereditary nonpolyposis colorectal cancer (HNPPC), anal canal cancer, acute or chronic inflammation, serious cardiovascular or cerebrovascular disease (such as acute coronary syndrome, chronic cardiac dysfunction, cerebral vascular accident), liver and kidney dysfunction, or other stress conditions. Healthy controls had no previous diagnoses of any major disease at health check-up and no history of cancer in the immediate relatives within three generations. Patients fasted overnight before the blood draw, upon which 5 ml of peripheral venous blood was collected in the early morning using an EDTA-K^2 anticoagulation vacuum blood collection tube.

Baseline characteristics

Baseline characteristics (sex and age) were obtained from adenocarcinoma and adenoma patients and healthy volunteers. Clinicopathological data (tumor location, type, diameter, histological grade, primary tumor invasion depth, vascular/perineural invasion, lymph node metastasis, and remote metastasis) were ascertained by an experienced pathologist. CRC patients were classified according to the TNM system based on the Amer-

ican Joint Committee on Cancer (*AJCC*) and Union for International Cancer Control (*UICC*) 2009 staging system for colorectal cancer (7th edition) [20].

CNRIP1 promoter methylation status in peripheral blood

Bisulfite modification was performed with an EpiTect bisulfite kit (QIAGEN, cat. no. 59104) completed as per the manufacturer's instructions. Briefly, DNA was dissolved in 3.6 mol/l sulfite solution, to which 800 µl of RNase-free water was added and mixed thoroughly, added into a 200 µl PCR tube, mixed at room temperature, and set aside. A Stratagene Mx3005P Real-Time PCR Detector (Agilent Technologies, Santa Clara, CA, USA) was used for DNA sulfite transformation, in a reaction totaling 5 h. The reactions were then incubated overnight at 20°C within the thermocycler.

The pyrosequencing: primers were designed using PyroMark Assay Design 2.0 (Qiagen, Germany). These primers were: CNRIP1 F1: 5′-GGTTATTTTTTTTAAGTTTTGGAAAGATT-3′; CNRIP1 R1: 5′-ATTTACCCACCACAATCCCCTTCA-3′; CNRIP1 S1: 5′-GGATTAGAGAGTAGTAGTGTTTA-3′ (5′-end modified by Biotin). Primers were synthesized by the Shenzhen BGI Company. The PCR was undertaken using an ABI PCR System 9700 (Applied Biosystems). The pyrosequencing reaction was undertaken using a PyroMark Q96 ID Pyrosequencing detector (QIAGEN). The methylation status of each locus was analyzed automatically by the Pyro Q-CpG software.

Cell cultures

SW620 (ATCC CCL227) and LoVo (ATCC CCL229) human colon cancer cell lines and the FHC normal human (ATCC CRL-1831) colon cell line were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, cat. no. 11965, Lot. 1177305) with 10% fetal bovine serum (Gibco, 10099-141-FBS) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a saturated humid atmosphere containing 5% CO_2. Cells were supplemented with 0.6 µg/ml trypsin (cat. no. T1426, Sigma, St. Louis, USA). Colon cancer cells were divided into four groups: 1) transfected with CNRIP1-shRNA (CNRIP1+ group; see *CNRIP1 overexpression*); 2) transfected with an empty vector (EV group); 3) treated with 5-azacytidine (5-azaC-treated group; see *CNRIP1 methylation suppression*); and 4) untreated and cultured in normal medium (control group).

CNRIP1 overexpression

CNRIP1+ and EV cells were transfected with either a *CNRIP1*-pcDNA3.0 plasmid or pcDNA3.0 plasmid, respectively. All transfections were performed using Lipofectamine 2000 reagent (cat. no. 52887; Invitrogen) used in accordance with the manufacturer's instructions. The primer was synthesized using a CNRIP1 CDS and the pcDNA3.0 (Invitrogen) vector sequence as a foundation. The length of the amplified fragment was 522 bp. The sequences of the CNRIP1-NotI F and CNRIP1-HindIII R primers were 5′-ATAAGAATGCGGCCGCAGACCCTCGCCCAGACATG-3′ and 5′-CCCAAGCTTAGTAGCATCAGAAAGAGCCAC-3′, respectively.

Suppression of *CNRIP1* methylation

Colon cancer cells (1.0×10^5/ well) were cultured in a 6-well plate containing DMEM with 10% FBS. The medium was replaced every 24 h with fresh medium containing 5 µM 5-azaC (EpiTect Bisulfite Kit, cat. no. 59104, QIAGEN, Hilden, Germany) for 72 h in total.

Levels of *CNRIP1* promoter methylation in colon cells

Genomic DNA was extracted and quantitated with a Biospin genomic DNA extraction kit (cat. no. BSC05S1). The EpiTect Bisulfite Kit (QIAGEN; cat. no. 59104) was used for bisulfite modification. The PCR primers (CNRIP1-F (5′-GAGTAGTAGTGTTTATTTTA-3′) and CNRIP1-R (5′-ACAATTTAAACTCCTCCTAAAAC-3′)) were synthesized by Shanghai Sangon Biotechnology. The PCR-amplified fragment length was 476 bp. TaKaRa Taq (Hot Start Version) was used for PCR amplification of the target gene. The PCR product was recovered with a Gel Extraction Kit (OMEGA) and was ligated into a cloning T-vector. The successfully cloned PCR product was selected and entrusted to Shanghai Sangon Biotechnology for sequencing. From the sequence data, the sequence similarity to the target sequences (%), C-T conversion efficiency (%), methylation statuses of each CG pair (dot plot), and methylation rate of each CG pair (%) were calculated.

Expression of CNRIP1 mRNA in colon cancer cells

The mRNA expression of CNRIP1 within SW620, LoVo, and FHC cell lines was determined with a Mx3005P Real-Time PCR Detector. β-actin was used as the housekeeping gene for qPCR. The primers were as follows: CNRIP1S (5′-GCATCCAGCCTAATGACGG-3′), CNRIP1AS (5′-TCAGTTCCAGTGGGACAAGC-3′), GAPDH forward (5′-TGTTCGTCATGGGTGTGAA-3′), and GAPDH reverse (5′-ATGGCATGGACTGTGGTCAT-3′). Polymerase chain reaction (PCR) primers were designed and synthesized using Primer Premier 6.0 software (Premier Biosoft International, Palo Alto, USA). SW620 and LoVo colon cancer cells were seeded into 6-well

plates (10^5 cells/well) and incubated for 24 h. RNA was extracted from cell lines using TRIzol reagent (Cat. 15596-018, Invitrogen, Darmstadt, Germany). SYBR Green fluorescence was measured at 72°C during the extension stage. The data were analyzed using the delta-delta cycle threshold (Ct) method [21]. All experiments were repeated in triplicate.

Expression of CNRIP1 protein in colon cancer cells

Western blotting was used to detect levels of CNRIP1 protein. Colon cancer cells were seeded into 6-well plates (10^6 cells/well) and incubated for 24 h. After cell lysis with RIPA buffer, the concentration of total protein was determined by BCA (Pierce TM BCA protein assay kit). 50 µg of cell lysates were electrophoresed at 100–120 V for 1.5 h, transferred to a polyvinylidene fluoride membrane, blocked with 5% non-fat milk, and incubated with 1 part anti-CNRIP1 antibody (Abcam, ab167087) and 2,000 parts PBS for 8 h at 25°C. Later, the Western blot was incubated with 1 part goat anti-rabbit IgG-HRP conjugate (BOSTER, cat. no. BA1054) and 20,000 parts PBS for 40 min at 25°C. The optical densities of protein bands were visualized with Image-Pro Plus 6.0 software using 1 part anti-β-actin antibody (Abcam, ab8245) and 400 parts PBS as an internal reference. Expression of proteins was determined in triplicate. The relative protein expression was calculated by dividing the gray value of the target protein with the gray value of the internal reference protein.

Proliferation ability of colon cancer cell lines

The proliferative ability of colon cancer cells was determined with a CCK-8 assay (YEASEN Cell Counting Kit-8, QF0025). Briefly, 5×10^4 colon cancer cells were seeded per microwell of a 96-well cell culture plate (Corning, 07-200-87). After incubating for 0, 6, 12, and 24 h, 10 µl of CCK-8 solution was added and incubated for 2 h. Following this, the OD_{450} was measured in triplicate using a microplate reader. A cell growth curve was plotted to calculate the proliferative ability of cells, which equaled the mean OD_{450} of experiment cells – mean OD_{450} of control cells.

Migration and invasion ability of colon cancer cell lines

The migration and invasive ability of colon cancer cells were determined by a Transwell assay consisting of a 24-well Transwell culture insert with polycarbonate membrane and 8.0 µm pores (BD FALCON, Franklin Lakes, USA). Briefly, the upper surface of the polycarbonate membrane of the Transwell insert was coated with 1 part Matrigel (50 mg/l) and 8 parts serum-free medium and was then allowed to air dry at 4°C. Colon cancer cells were trypsinized, centrifuged at 12,000 g for 10 min, and the supernatant discarded. The cells were then resuspended in serum-free medium, counted, and diluted to a density of 5×10^5/ml. A total of 500 µl of DMEM (Life Technologies, cat. no. 11965, lot no. 1177305) was added to each well. Next, 100–200 µl of cell suspension was transferred into the upper compartment of the cell culture insert and incubated at 37°C for 12–48 h. Next, the most superficial cells were wiped with a wet cotton swab and the upper compartment was removed. The polycarbonate membrane was fixed with 4% paraformaldehyde (CAS 30525-89-4, Santa Cruz Biotechnology) for 15 min at 25°C and then was inverted and stained with 0.1% crystal violet solution (HT90132, Sigma, USA) for 15 min. Stained cells were counted under a microscope (Zeiss, Axio Imager2, 400×). Six visual fields per chamber were selected for imaging. The number of migrated cells on the lower surface of the polycarbonate membrane was counted within each field. The numbers of cells per group were calculated and were then adjusted to the number in the control group. An uncoated polycarbonate membrane was used to detect the baseline migration ability of colon cancer cells. Finally, the cell invasion/migration inhibition ratio was calculated with the following formula: ((invading/migrated cells in the control group (n) – invading/ migrating cells in the experimental group (n))/number of invading/migrated cells in the control group) × 100%.

Statistical analysis

All data are presented as the mean ± standard deviation (SD). Data were analyzed using SPSS V 19.0 software (IBM, Armonk, USA). The χ^2 test was used to analyze the correlation between *CNRIP1* methylation and clinicopathological features. Student's *t*-test was used to analyze the proliferation and invasion/migration of colon cancer cells. Pearson's correlation analysis was used to analyze the correlation between *CNRIP1* expression and proliferation of colorectal cancer cells. A p-value < 0.05 was considered statistically significant.

Results

Levels of *CNRIP1* promoter methylation

Variations in the methylation level at numerous loci within the *CNRIP1* promoter region were assessed in blood drawn from colorectal adenocarcinoma and adenoma patients and healthy volunteers. There were 8 CpG sequences (at positions 2216, 2226, 2231, 2245, 2254, 2273, 2286, and 2325, denoted with a double-underline in the fol-

lowing sequence, respectively) within the *CNRIP1* promoter region: 5′-GAGCAGCAGTGTCCACCTCACGTAGCTGGCCGAGGCGGTATCCAGATTCCGGGGGTCTCGCTCCTTGGCATAGTGGTCGCTTAACTCCAGCGCCTTCCCAGTGCCTCCCAAACCTCTCCTCCTCCTGCCCGGGCTGTTCTAGGAGGAGCCTAAATGTC-3′.

The methylation levels in patients with colorectal adenocarcinoma, colorectal adenoma, and healthy volunteers were 85.50 ±8.24%, 81.33 ±5.81%, and 63.66 ±3.61%, respectively. Sequencing of CpG islands revealed differences in the methylation levels at loci 2216, 2226, 2231, 2245, and 2254 within the *CNRIP1* promoter region, which was most marked at locus 2245 (Figure 1). Compared with healthy volunteers, the methylation levels in the sample of patients with colorectal adenocarcinoma and colorectal adenoma were significantly higher ($p < 0.01$; Table I). There were no significant differences between patients with colorectal adenocarcinoma and colorectal adenoma ($p > 0.05$; Table I).

Association between *CNRIP1* promoter region methylation status and clinicopathological characteristics of colorectal adenocarcinomas

The correlations between the methylation status within the *CNRIP1* promoter region and clinicopathological characteristics of diagnosed adenocarcinomas were assessed. Based on the methylation levels at locus 2245, patients with adenocarcinoma and adenoma and healthy controls were stratified into methylation ≥ 80% and methylation < 80% groups, and χ^2 analysis was performed (Table II).

Verification of *CNRIP1* overexpression in colon cancer cells

BLAST comparative analysis of the *CNRIP1*-transfected clone sequence data was conducted with the sequences listed in the database curated by the National Center for Biotechnology Information (NCBI) (Figure 2). The results show that the sequences were consistent with one another, indicating that the CNRIP1 CDS was successfully cloned with the PCDNA3.0 vector.

Levels of *CNRIP1* promoter methylation in colon cancer cell lines

CNRIP1 promoter methylation levels of colon cancer cell lines before and after 5-azaC treatment were analyzed. The average methylation levels within CpG sequences of the *CNRIP1* promoter region were 96.9% (75% at locus 2216, 100% at 2226, 100% at 2231, 100% at 2245, 100% at 2254, 100% at 2273, 100% at 2286, and 100% at 2325) before 5-azaC treatment and 47.5% (60% at locus 2216, 60% at 2226, 60% at 2231, 40% at 2245, 40% at 2254, 40% at 2273, 40% at 2286, and 40% at 2325) after 5-azaC treatment (Figure 3).

Expression of CNRIP1 mRNA in colon cancer cells

The results of qPCR show that CNRIP1 mRNA expression was significantly lower ($p < 0.05$) within SW620 colon cancer cells (0.51 ±0.03) than in the LoVo colon cancer (1.00 ±0.10) and FHC normal colon (0.79 ±0.06) cell lines (Figure 4). The CNRIP1 mRNA expression level in SW620 cells after 5-azaC treatment was significantly higher (2.72 ±0.33; $p < 0.05$) than within untreated SW620 cells (1.00 ±0.04; Figure 5). The mRNA expression significantly increased ($p < 0.05$) within cells transfected with a *CNRIP1*-overexpression plasmid (68.30 ±2.08) relative to SW620 cells within the EV group (1.00 ±0.04; Figure 6).

Expression of CNRIP1 protein in colon cell lines

The level of CRNIP1 protein expression was determined by Western blot analysis. The CNRIP1 protein expression level within the SW620 colon cancer cell line was significantly lower (0.151 ±0.03; $p < 0.05$) than within the LoVo colon cancer (0.287 ±0.05) and normal FHC colon cell lines (0.711 ±0.04; Figure 7). Treatment of SW620 cells with 5-azaC resulted in a significantly increased ($p < 0.05$) level of CNRIP1 protein expression (0.900 ±0.05) relative to untreated SW620 cells (0.329 ±0.02; Figure 8). The level CNRIP1 protein expression was significantly higher ($p < 0.05$) within cells transfected with a *CNRIP1*-overexpression plasmid (0.615 ±0.05) relative to control SW620 cells within the EV group (0.177 ±0.03; Figure 9).

Proliferation of colon cancer cells

The proliferative potential was compared between 5-azaC-treated and untreated SW620 colon cancer cells (Figure 10) and between SW620 cells transfected with a *CNRIP1*-overexpression plasmid and SW620 cells transfected with an empty vector (Figure 11). The proliferative ability of SW620 cells at 96 h post 5-azaC treatment was significantly lower (0.792 ±0.05 OD_{450}; $t = 25.812$; $p = 0.001$) than the same cells before 5-azaC treatment (1.443 ±0.02 OD_{450}). Moreover, the Pearson correlation showed that proliferative ability of treated SW620 cells positively correlated with the duration of 5-azaC treatment ($r = 0.931$; $p = 0.021$). Meanwhile, the proliferative ability of SW620 cells transfected with *CNRIP1*-overexpression plasmid at 96 h was significantly lower (0.95 ±0.37 OD_{450}; $t = 5.382$; $p < 0.001$) than SW620 cells transfected with a control vector (1.29 ±0.61 OD_{450}).

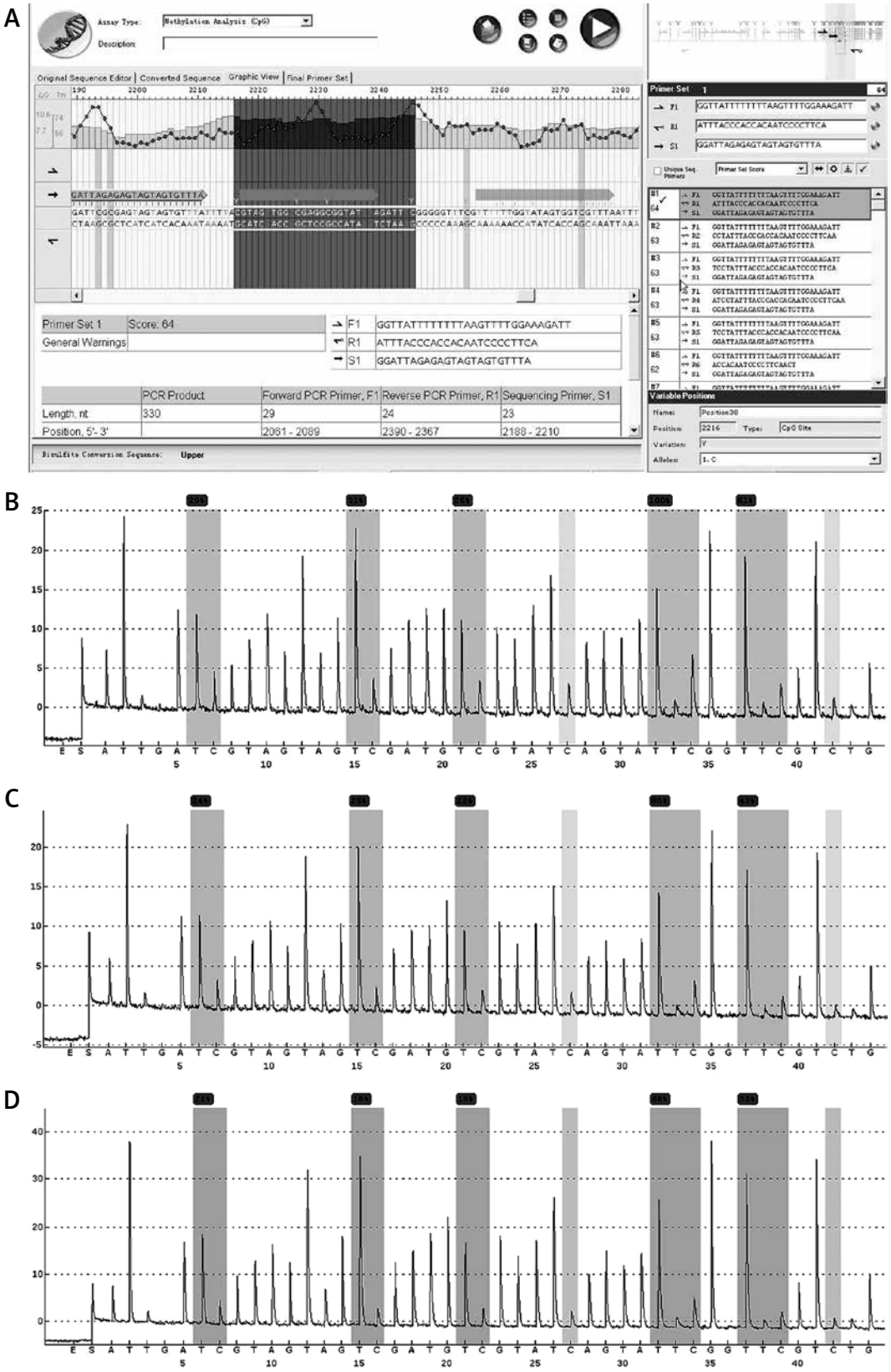


Figure 1. *CNRIP1* promoter CpG island methylation pyrosequencing map of peripheral blood. **A** – The pyrosequencing primers were designed using PyroMark Assay Design 2.0, **B** – colorectal adenocarcinoma patients, **C** – colorectal adenoma patients, **D** – healthy volunteer (Y is a degenerate base corresponding to T and C; methylation level is indicated by percentage). Methylation levels in patients with colorectal adenocarcinoma, colorectal adenoma, and healthy volunteers were 85.50 ±8.24%, 81.33 ±5.81%, and 63.66 ±3.61%, respectively. Sequencing of CpG islands revealed differences in the methylation levels at loci 2216, 2226, 2231, 2245, and 2254 within the *CNRIP1* promoter region, and it was most marked at locus 2245

Table I. CNRIP1 promoter methylation levels in peripheral blood sample

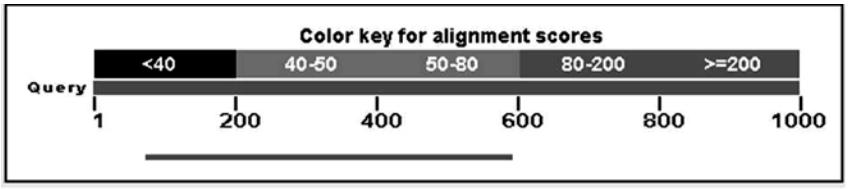
Variable	*N*	CNRIP1 promoter methylation levels (%)	*t* value	*P*-value
CRC patients	50	85.50 ±8.24	5.94	0.008*
Colorectal adenoma patients	20	81.33 ±5.81	6.50	0.000#
Healthy volunteers	20	63.66 ±3.61	2.10	0.089▲

**p < 0.05 (CRC patients compared with healthy volunteers); #p < 0.05 (colorectal adenoma patients compared with healthy volunteers); ▲p > 0.05 (CRC patients compared with colorectal adenoma patients).*

Table II. Correlation between CNRIP1 promoter methylation and clinicopathological characteristics of colorectal adenocarcinoma

Clinicopathological parameters	*N* = 50	CNRIP1 methylation		χ^2 value	*P*-value
		≥ 80%	< 80%		
Gender:					
Male	28	19	9	0.001	0.981
Female	22	15	7		
Age [years]:					
≥ 60	31	24	7	3.326	0.068
< 60	19	10	9		
Tumor location:					
Ascending colon	19	14	5	1.283	0.864
Transverse colon	3	2	1		
Descending colon	4	3	1		
Sigmoid colon	6	3	3		
Rectum	18	12	6		
General classification:					
Mass type	8	5	3	0.139	0.933
Infiltrating type	19	13	6		
Ulcerative type	23	16	7		
Tumor diameter [cm]:					
≤ 3	21	8	13	14.880	< 0.001*
> 3	29	26	3		
Differentiation:					
High	9	3	6	6.941	0.031*
Medium	23	16	7		
Low	18	15	3		
Depth of invasion:					
Tis	23	10	13	11.847	0.003*
T1 + T2	15	13	2		
T3 + T4	12	11	1		
Lymph node metastasis:					
N0	33	19	14	4.847	0.028*
N1 + N2	17	15	2		
Distant metastasis:					
M0	44	29	15	0.737	0.391
M1	6	5	1		
TNM staging:					
I + II	27	13	14	10.630	0.001*
III + IV	23	21	2		

**p < 0.05.*



Sequences producing significant alignments

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Alignments Download Graphics

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	None provided	944	944	52%	0.0	99%	Query_245001

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Sequence ID: lcl|Query_245001 Length: 522 Number of Matches: 1

Range 1: 1 to 520 Graphics ▼ Next Match ▲ Previous M

Score	Expect	Identities	Gaps	Strand
944 bits(511)	0.0	517/520(99%)	0/520(0%)	Plus/Minus

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                                                           in the NCBI database
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Figure 2. Verification of *CNRIP1* overexpression in colon cancer cells. BLAST comparative analysis of the *CNRIP1*-transfected clone sequence data was conducted with the sequences listed in the database curated by the National Center for Biotechnology Information (NCBI). Sequences were consistent with one another, thereby indicating that the *CNRIP1* CDS was successfully cloned via our PCDNA3.0 vector

Invasion and migration of colon cancer cells

We then compared the invasive and migration ability of 5-azaC treated and untreated SW620 colon cancer cells and SW620 cells transfected with a *CNRIP1*-overexpression plasmid and SW620 cells transfected with an empty vector. Treatment of SW620 cells with 5-azaC for 96 h significantly decreased their invasive (57.33 ±15.92 cells/well) and migration ability (79.83 ±9.50 cells/well), when compared to the invasiveness (150.00 ±11.80 cells/well; t = 8.50, p = 0.001; Figure 12) and migration (154.33 ±17.48 cells/well; t = 8.18, p = 0.001; Figure 13) of untreated SW620 cells. Furthermore, transfection of SW620 cells with a *CNRIP1*-overexpression plasmid significantly reduced their invasiveness (94.00 ±16.24 cells/well) and migration ability (101.5 ±13.89 cells/well), when compared to the invasiveness (171.83 ±13.93 cells/well; t = 9.642, p = 0.000; Figure 14) and migration ability (134.33 ±5.72 cells/well; t = 5.126, p = 0.004; Figure 15) of SW620 colon cancer cells transfected with an empty vector.

Discussion

The present study found that the methylation status at locus 2245 within the CpG island re-

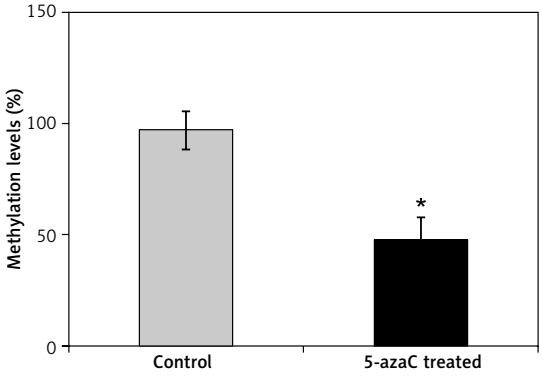


Figure 3. *CNRIP1* promoter methylation level in colon cancer cell lines. *CNRIP1* promoter methylation levels of colon cancer cell lines before and after 5-azaC treatment were analyzed. The average methylation levels within CpG sequences of the *CNRIP1* promoter region were 96.9% (75% at locus 2216, 100% at 2226, 100% at 2231, 100% at 2245, 100% at 2254, 100% at 2273, 100% at 2286, and 100% at 2325) before 5-azaC treatment and 47.5% (60% at locus 2216, 60% at 2226, 60% at 2231, 40% at 2245, 40% at 2254, 40% at 2273, 40% at 2286, and 40% at 2325) after 5-azaC treatment

**p < 0.05 (control vs. 5-azaC treated).*

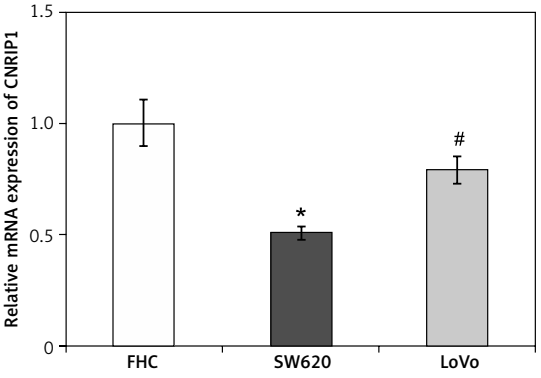


Figure 4. CNRIP1 mRNA expression in colon cells. CNRIP1 mRNA expression was significantly lower (p < 0.05) within the SW620 colon cancer cell line (0.51 ±0.03) than in the LoVo colon cancer (1.00 ±0.10) and FHC normal colon (0.79 ±0.06) cell lines

**p < 0.05 (SW620 vs. FHC), #p < 0.05 (SW620 vs. LoVo).*

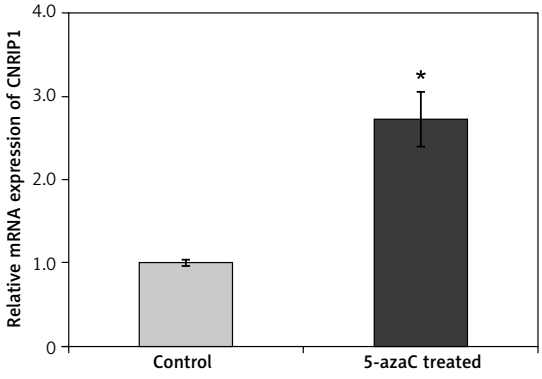


Figure 5. CNRIP1 mRNA expression levels in colon cancer cells before and after 5-azaC treatment. The CNRIP1 mRNA expression level in SW620 cells after 5-azaC treatment was significantly higher (2.72 ±0.33; p < 0.05) than within SW620 cells not treated with 5-azaC (1.00 ±0.04)

**p < 0.05 (control vs. 5-azaC treated).*

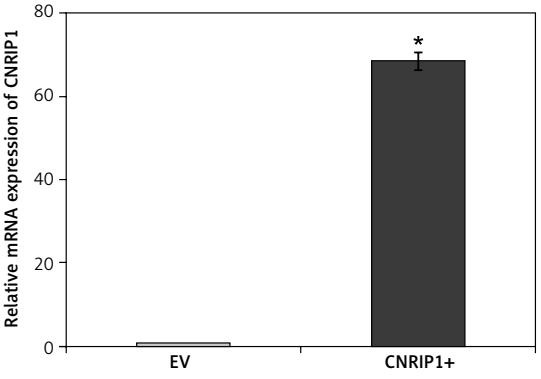


Figure 6. CNRIP1 mRNA expression levels in colon cancer cells before and after transfection with a *CNRIP1*-overexpression plasmid. The mRNA expression significantly increased (p < 0.05) within cells transfected with a *CNRIP1*-overexpression plasmid (68.30 ±2.08) relative to SW620 cells within the EV group (1.00 ±0.04)

**p < 0.05 (CNRIP1 vs. EV).*

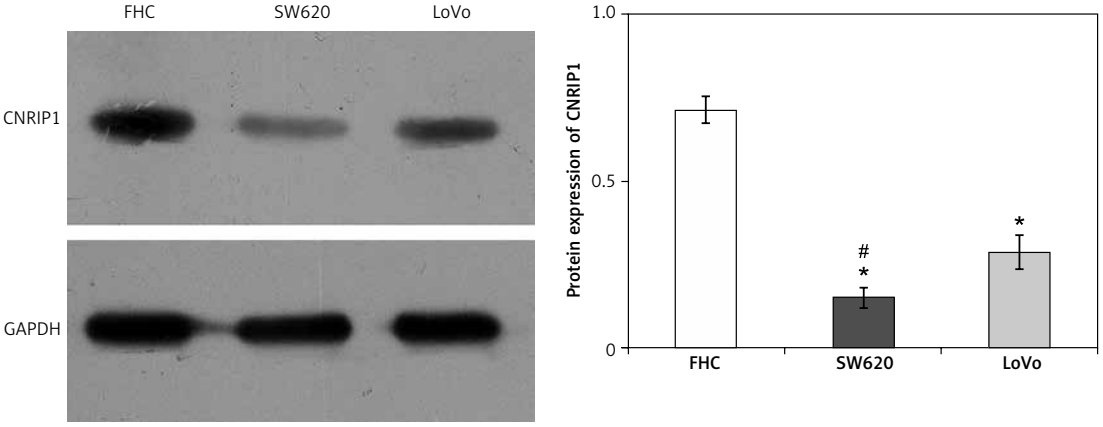


Figure 7. CNRIP1 protein expression levels within colon cell lines. The CNRIP1 protein expression level within the SW620 colon cancer cell line was significantly lower (0.151 ±0.03; $p < 0.05$) than within the LoVo colon cancer (0.287 ±0.05) and normal FHC colon cell lines (0.711 ±0.04). CRNIP1 protein expression was determined by Western blotting

**p < 0.05 (SW620 vs. FHC), #p < 0.05 (SW620 vs. LoVo).*

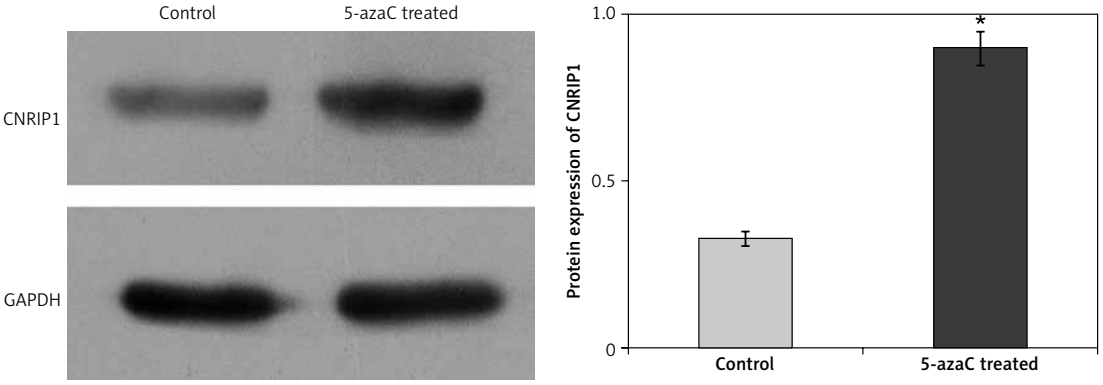


Figure 8. CNRIP1 protein expression levels within colon cancer cell lines treated with 5-azaC. Treatment of SW620 with 5-azaC resulted in a significantly increased ($p < 0.05$) level of CNRIP1 protein expression (0.900 ±0.05) relative to untreated SW620 cells (0.329 ±0.02)

**p < 0.05 (5-azaC treated vs. control).*

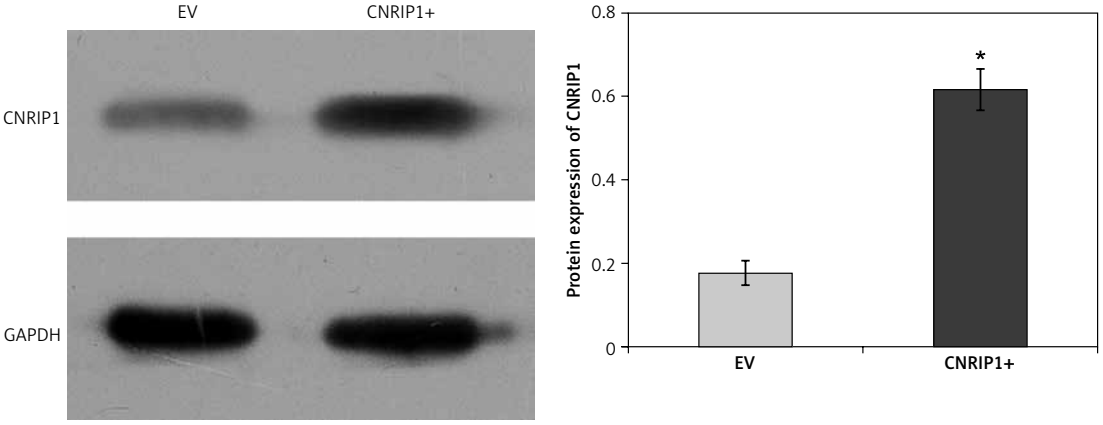


Figure 9. CNRIP1 protein expression levels within colon cancer cell lines treated with a *CNRIP1*-overexpression plasmid. The level of CNRIP1 protein expression was significantly higher ($p < 0.05$) within cells transfected with a *CNRIP1*-overexpression plasmid (0.615 ±0.05) relative to control SW620 cells within the EV group (0.177 ±0.03)

**p < 0.05 (CNRIP1+ vs. EV).*

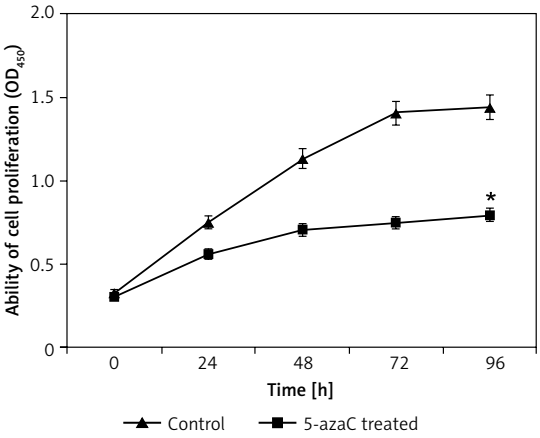


Figure 10. Proliferation of colon cancer cells treated with 5-azaC. The proliferative ability of SW620 cells at 96 h after 5-azaC treatment was significantly lower (0.792 ±0.05 OD_{450}; t = 25.812; p = 0.001) than the same cells before 5-azaC treatment (1.443 ±0.02 OD_{450})

**p < 0.05 (5-azaC treated vs. control).*

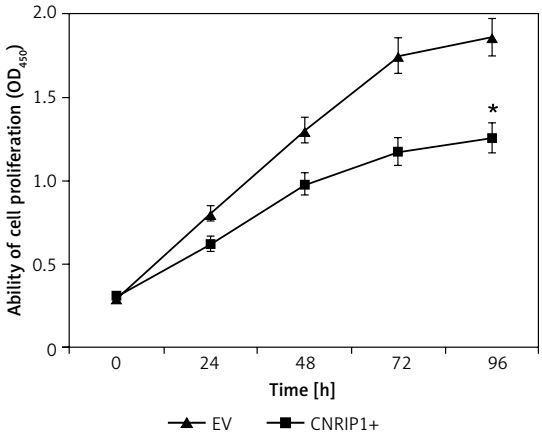
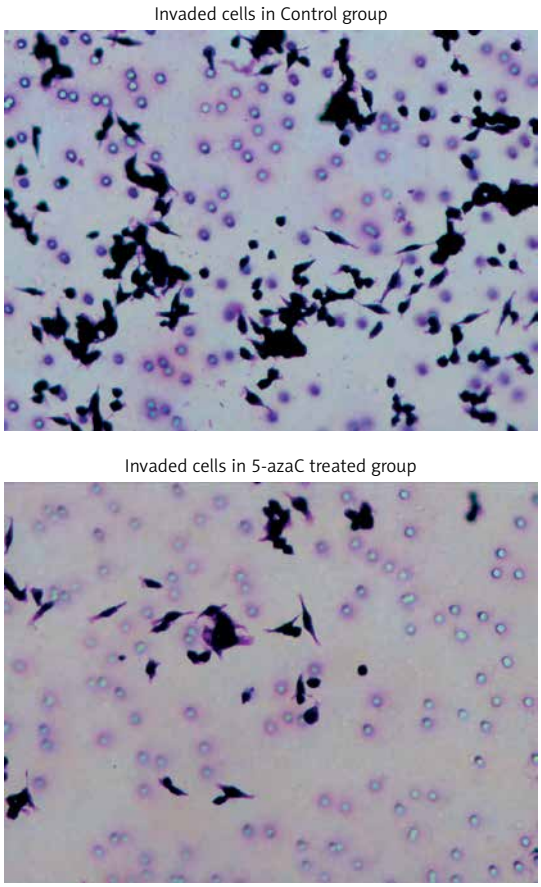


Figure 11. Proliferation of cancer cells transfected with a *CNRIP1*-overexpression plasmid. The proliferative ability of SW620 cells transfected with a *CNRIP1*-overexpression plasmid was significantly lower (0.95 ±0.37 OD_{450}; t = 5.382; p < 0.001) than SW620 cells transfected with a control vector (1.29 ±0.61 OD_{450})

**p < 0.05 (CNRIP1+ vs. EV).*



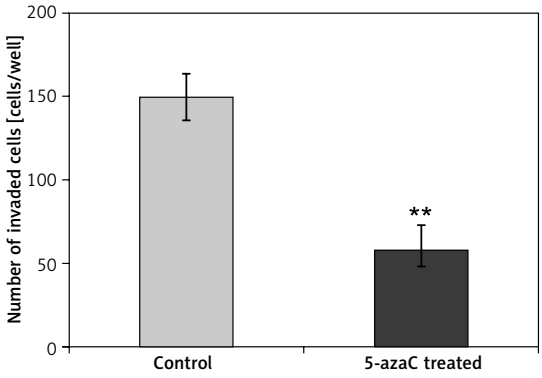


Figure 12. Invasion of colon cancer cells treated with 5-azaC. Treatment of SW620 cells with 5-azaC for 96 h significantly decreased their invasiveness (57.33 ±15.92 cells/well) when compared to the invasiveness of untreated SW620 cells (150.00 ±11.80 cells/well; t = 8.50; p = 0.001)

***p < 0.01 (5-azaC treated vs. Control).*

gion of the *CNRIP1* promoter region was greater from the peripheral blood of patients with colorectal adenocarcinoma than within healthy controls. Furthermore, the methylation level at the 2245 locus significantly correlated with tumor diameter, the depth of primary tumor invasion, TNM stage, tumor grade, and lymph node metastasis, therefore suggesting that the degree of methylation level may have prognostic value. The results also show that the methylation status of locus 2245 was higher within colorectal adenoma patients, thereby highlighting its potential

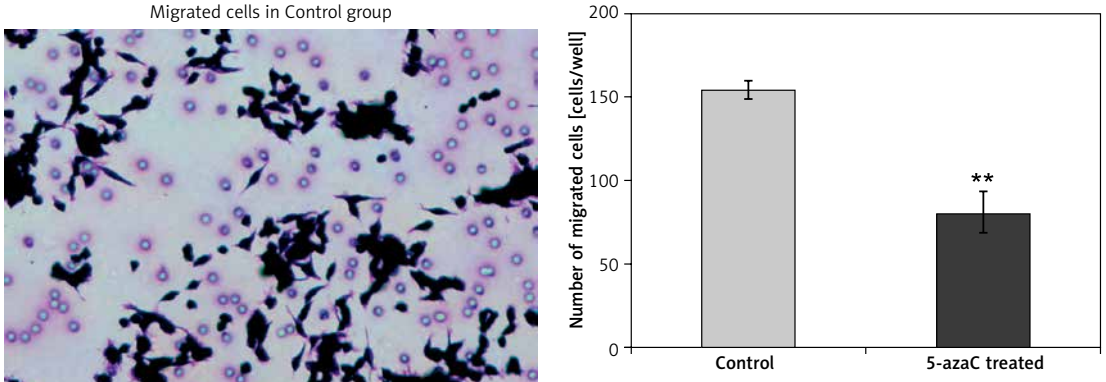


Figure 13. Migration of colon cancer cells treated with 5-azaC. Treatment of SW620 cells with 5-azaC for 96 h significantly decreased their migration ability (79.83 ±9.50 cells/well) when compared to the migration of untreated SW620 cells (154.33 ±17.48 cells/well; $t = 8.18$; $p = 0.001$)

***p < 0.01 (5-azaC treated vs. Control).*

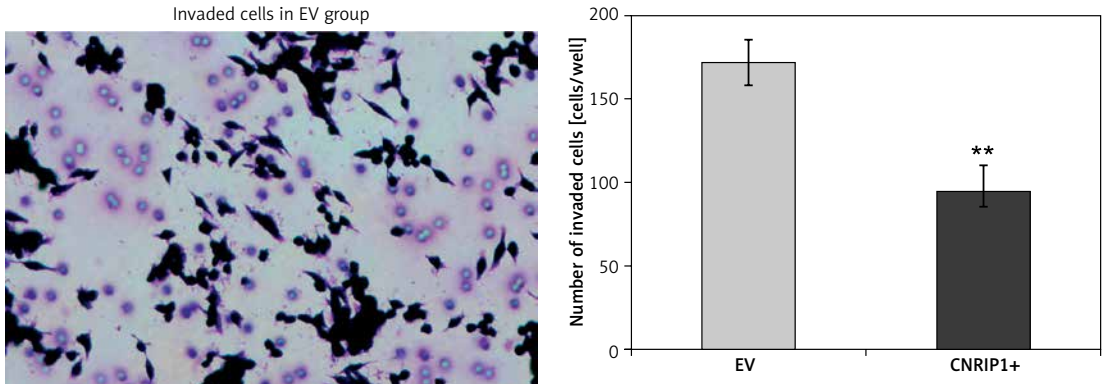


Figure 14. Invasion of colon cancer cells transfected with a *CNRIP1*-overexpression plasmid. Transfection of SW620 cells with a *CNRIP1*-overexpression plasmid significantly reduced their invasiveness (94.00 ±16.24 cells/well) when compared to the invasiveness of SW620 colon cancer cells transfected with an empty vector (171.83 ±13.93 cells/well; $t = 9.642$; $p < 0.001$)

***p < 0.01 (CNRIP1+ vs. EV).*

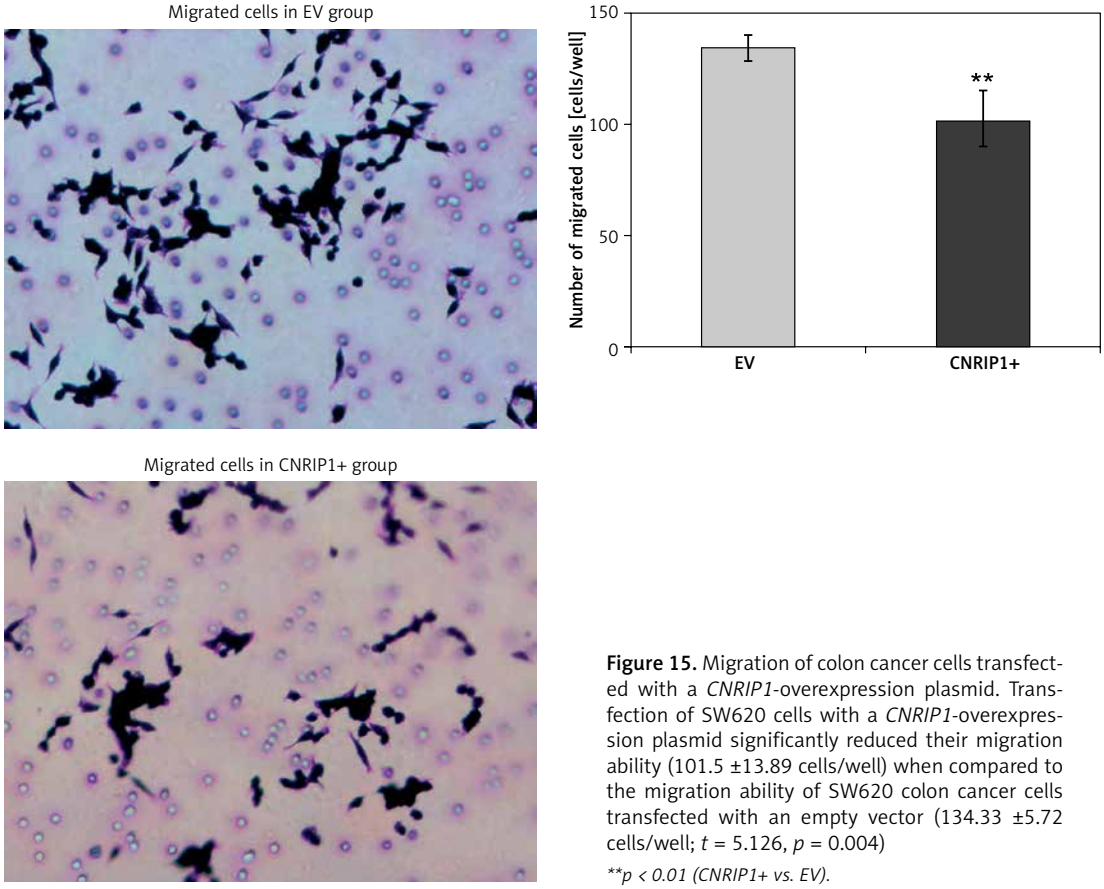


Figure 15. Migration of colon cancer cells transfected with a *CNRIP1*-overexpression plasmid. Transfection of SW620 cells with a *CNRIP1*-overexpression plasmid significantly reduced their migration ability (101.5 ±13.89 cells/well) when compared to the migration ability of SW620 colon cancer cells transfected with an empty vector (134.33 ±5.72 cells/well; $t = 5.126$, $p = 0.004$)

***$p < 0.01$ (CNRIP1+ vs. EV).*

as a screening tool. Accordingly, screening the peripheral blood drawn from the general population for the methylation status at locus 2245 in the CpG island region of the *CNRIP1* promoter may be helpful for detecting the precancerous colorectal adenoma, which can be excised before developing into cancer, or non-advanced adenocarcinomas, which are highly treatable and have more favorable outcomes.

To date, there have been few studies investigating the role of *CNRIP1* and its methylation in dysplastic and normal human body tissues. In order to verify the effect of *CNRIP1* methylation on the proliferative, invasive and migration ability of colorectal cancer cells, we used qPCR and Western blotting to select a low *CNRIP1* expression SW620 colon cancer cell line for cell function assays. We first used 5-azaC to suppress methylation in treated SW620 cells. We found that, after 5-azaC treatment, CNRIP1 mRNA expression and protein synthesis in SW620 cells were significantly increased, whereas the methylation level of the *CNRIP1* promoter in SW620 cells was significantly reduced ($p < 0.05$). CCK-8 and Transwell assays were then used to detect the proliferative, invasive and migration abilities of colon cancer cells, wherein we found that the proliferative, invasion and migration abilities of SW620 cells were significantly decreased ($p < 0.05$) after the treatment of 5-azaC-induced methylation suppression, and that the performance of cells in such assays correlated with the duration of 5-azaC treatment. Our study results suggest that hypomethylation of the *CNRIP1* promoter region significantly decreases the proliferative, invasive and migration abilities of SW620 colon cancer cells. Could *CNRIP1* promoter demethylation be a potential therapeutic for treating colorectal cancer?

In order to verify the effect of *CNRIP1* overexpression on the activity of colorectal cancer cells, the *CNRIP1*-overexpression lentiviral plasmid was constructed and transfected into SW620 colon cancer cells. We found that, compared with the colon cancer cells transfected with an empty vector, the cell proliferative, invasive and migration ability of colon cancer cells overexpressing *CNRIP1* were significantly reduced ($p < 0.05$). Our results suggest that *CNRIP1* could be a tumor suppressor gene and that inducing *CNRIP1* overexpression could be developed into a valuable colorectal cancer therapeutic.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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