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Epigenetic Reactivation of the E-Cadherin (*CDH1*) Gene and Induction of Autophagy by 5-Azacytidine in Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is a serious neoplasm associated with high mortality rates. Aberrant epigenetic alterations are involved in multiple stages of tumor progression. The FDA has approved 5-azacytidine (5-aza) for the treatment of various cancers, including leukemia and myelodysplastic syndromes (MDS). Previous studies have demonstrated that E-cadherin (*CDH1*) expression is suppressed in CRC; however, the regulatory mechanisms underlying this suppression remain unclear.This study aimed to examine the potential therapeutic effects of 5-aza in CRC using the human LS513 cell line. Cell viability was assessed using the MTT assay, while cell proliferation and autophagy were evaluated by flow cytometry. *CDH1* expression levels were analysed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) and Western blotting, and methylation-specific PCR (MSP-PCR) was used to determine the methylation pattern of the *CDH1* promoter region.5-aza significantly decreased LS513 cell viability in a dose-dependent manner ($P < 0.001$). Furthermore, 5-aza treatment induced cell cycle arrest at the G2/M phase, induced autophagy, and upregulated *CDH1* expression through demethylation of the hypermethylated *CDH1* promoter.

Keywords: Colorectal cancer; Epigenetic therapy; DNA methylation; 5-azacytidine; *CDH1*; Autophagy; Cell cycle arrest.

1. Introduction

Colorectal cancer ranks among the leading causes of cancer-related deaths in both genders worldwide, despite advancements in screening and treatment. Its prevalence has steadily increased, particularly in countries with moderate to high development levels and among younger populations [1]. CRC develops through a combination of genetic mutations and epigenetic modifications in cellular DNA, transforming normal epithelial cells into adenocarcinoma. These alterations may confer migratory and invasive properties on cancer cells, enabling metastasis to distant sites [2,3].DNA hypermethylation can be therapeutically reversed using DNA methyltransferase inhibitors. The association between tumor suppressor gene (TSG) hypermethylation and CRC progression has been previously reported [4–7]. Reactivation of TSGs silenced by DNA hypermethylation represents a promising strategy for cancer treatment through epigenetic therapy. Previous studies have demonstrated that 5-aza can upregulate hypermethylated TSGs, enhancing their intrinsic anticancer activity.

In cancer cells, hypermethylation primarily occurs in promoter regions of genes encoding DNA repair proteins, adhesion molecules, and TSGs [8], such as the *CDH1* gene, that is associated with tumor invasiveness and progression [9,10]. *CDH1* dysfunction plays a critical role in tumor progression, as it is an essential component of epithelial cell adhesion [11,12]. Several studies have indicated that *CDH1* hypermethylation negatively correlates with mRNA expression and promotes tumor growth, while suppression of *CDH1* expression facilitates metastasis [13]. The loss of differentiation observed in various carcinomas, including CRC, has been linked to *CDH1* downregulation [14,15].

Given the reversible nature of epigenetic changes, there is growing interest in employing epigenetic therapy to correct aberrant DNA methylation. Consequently, DNA methyltransferase enzymes (DNMTs), which play a central role in epigenetic regulation, have received considerable attention [16,17]. This study aimed to investigate the effects of 5-aza on cell viability, *CDH1* reactivation, cell cycle regulation, and autophagy, as well as its potential as a therapeutic option for CRC.

2. Materials and Methods

Chemicals

5-Azacytidinewas purchased from Sigma-Aldrich (St. Louis, MO, USA). RPMI-1640 medium was obtained from Sigma-Aldrich (St. Louis, MO, USA), and dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany).

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Experimental cell line

The human LS513 cell line (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were maintained in a humidified incubator at 37 °C with 5% (v/v) CO_2. LS513 cells were seeded in six-well plates at a density of 1×10^6 cells/well in complete medium and incubated overnight. Based on the calculated IC_{50} value of 63 µM, the cells were treated with 5-aza at concentrations of 10, 50, and 100 µM for 48 h. Throughout the experiment, the culture medium containing the drug was replaced daily.

Cytotoxicity measurement

The viability of LS513 cells was evaluated using the MTT assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells per 100 µL of complete medium and incubated overnight. Subsequently, the cells were treated with various concentrations of 5-aza(3, 10, 30, 100, and 300 µM) for 48 h. After treatment, the medium was removed, and the cells were incubated with MTT solution for 4 h at 37 °C. The resulting formazan crystals were then dissolved in 100 µL of dimethyl sulfoxide (DMSO), and absorbance was measured at 490 nm using a microplate reader (BMG LABTECH FLUOstar Omega, Ortenberg, Germany) to determine cell viability.

Detection of gene expressions using RT-qPCR

To analyse*CDH1* mRNA expression, LS513 cells were treated with 5-aza at concentrations of 10, 50, and 100 µM. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and its concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized from the isolated RNA using a reverse transcription kit. Quantitative real-time PCR (RT-qPCR) was performed with SYBR Green qPCR Master Mix and specific primers (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control, and relative gene expression levels were calculated using the $2^{\wedge-\Delta\Delta CT}$ method.

Table 1: Primer Sequence for RT-qPCR

Gene	Primer sequence	Tm°C	Product size (bp)
CDH1	F: ACACGTAGCAGTGACGAATG	58.3 °C	115
	R: TTCAGGAGGCACAAAGATGG	57.7 °C	
GAPDH	F: CTCAACTACATGGTTTACA	49.5°C	113
	R: AAGATGGTGATGGGATTT	50.4°C	

Western blotting

Total protein was extracted using RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 1% SDS, 1 mM EDTA, and a protease and phosphatase inhibitor cocktail). Equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat dry milk in TBST for 1 h at room temperature and then incubated overnight at 4 °C with primary antibodies against *CDH1* (FAGUS, UK) and β-actin (Invitrogen, USA). After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized using an enhanced chemiluminescence (ECL) detection system (Thermo Fisher Scientific, USA).

Methylation analysis using MSP-PCR

Genomic DNA was extracted from treated and untreated LS513 cells using the Genomic DNA Extraction Kit (Qiagen, Hilden, Germany). Bisulfite modification was performed according to the manufacturer's instructions using the EpiJET Bisulfite Conversion Kit (Thermo Fisher Scientific, Germany). Methylation-specific PCR (MSP) was employed to detect aberrant methylation patterns in the *CDH1* promoter region using primers specific for methylated (M) and unmethylated (U) DNA. The primer sequences were as follows: *CDH1* (M) forward, 5′-GTAAAGTATTTGTGAGTTT-GCGG-3′ and reverse, 5′-ACCTACAACAACAACAACAACG-3′; *CDH1* (U) forward, 5′-GAATTGTAAAGTATTT-GTGAGTTTGTG-3′ and reverse, 5′-AAAAC-CTACAACAACAACAACAACA-3′. Primers were designed using the MethPrimer database (http://www.urogene.org/methprimer2/). PCR amplification was performed with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 10 min. PCR products were separated on a 2% agarose gel prepared in TBE buffer and visualized under UV illumination after ethidium bromide staining.

Cell cycle analysis

Flow cytometry was used to analyse the cell cycle and assess the proliferation of LS513 cells following treatment with 5-aza at concentrations of 10, 50, and 100 µM. Briefly, 1×10^5 cells were washed with ice-cold phosphate-buffered saline (PBS) and fixed in 70% ethanol overnight at 4 °C. The fixed cells were then stained with propidium iodide (PI) and analysed using a flow cytometer (ACEA NovoCyte, USA). PI fluorescence was detected at 535/617 nm, and data were processed using ACEA NovoExpress software (ACEA Biosciences, USA).

Autophagy assessment

Autophagy was evaluated using the Cyto-ID Autophagy Detection Reagent (Abcam, Cambridge, UK) in LS513 cells treated with 5-aza for 48 h. Following treatment, cells were stained with Cyto-ID Green dye according to the manufacturer's instructions, and fluorescence was analyzed by flow cytometry. Green and orange fluorescence signals were recorded in the FL1 and FL2 channels, respectively.

Statistical analysis

All experiments were performed in triplicate ($n = 3$), and the results are presented as the mean ± standard deviation (SD). Differences among groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Statistical analyses were performed using GraphPad Prism software (version 9.5.0 for Windows; GraphPad Software, USA). A p-value < 0.05 was considered statistically significant.

3. Results

5-aza suppressed the viability of LS513 cells

The inhibition of cell viability is a key characteristic of most antitumor agents. The MTT assay was performed to evaluate the effect of 5-aza on LS513 cell viability. The results revealed an IC_{50} value of 63 µM. Treatment with lower concentrations of 5-aza did not significantly affect cell viability; however, 5-aza reduced LS513 cell viability in a concentration-dependent manner, with a significant inhibitory effect observed at doses of 10 µM and above (Figure 1).

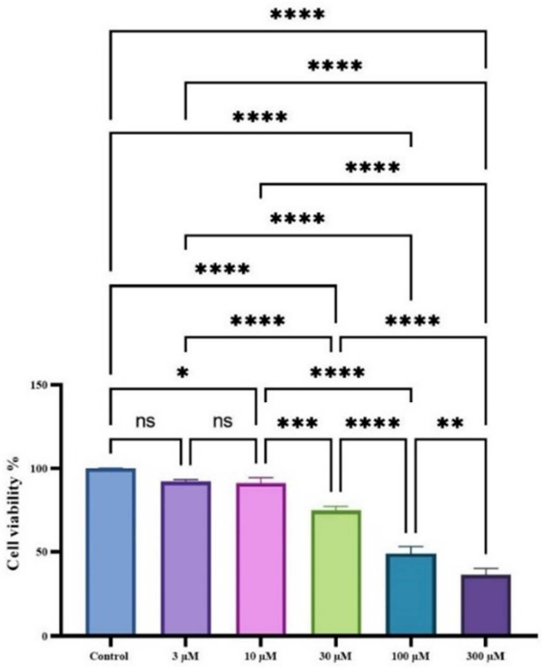

Figure 1: Effect of 5-azacytidine on LS513 cell viability. Cells were treated with increasing concentrations of 5-aza (3–300 µM) for 48 h, and viability was measured using the MTT assay. Data are presented as mean ± SD from three independent experiments (n = 3). Statistical differences were determined using one-way ANOVA followed by Tukey's multiple comparison test.*p < 0.05 (*), p < 0.01 (**), p < 0.001 (***)* vs. untreated control.

5-aza upregulates the *CDH1* gene expression in CRC

Based on the MTT assay results, LS513 cells were treated with 5-aza at concentrations of 10, 50, and 100 µM. RT-qPCR analysis revealed that 5-aza significantly upregulated *CDH1* mRNA expression compared with the control group (Figure 2A). Western blot analysis confirmed a similar increase at the protein level in LS513 cells (Figure 2B, C). These findings indicate that 5-aza enhances *CDH1* expression in LS513 cells relative to the untreated control.

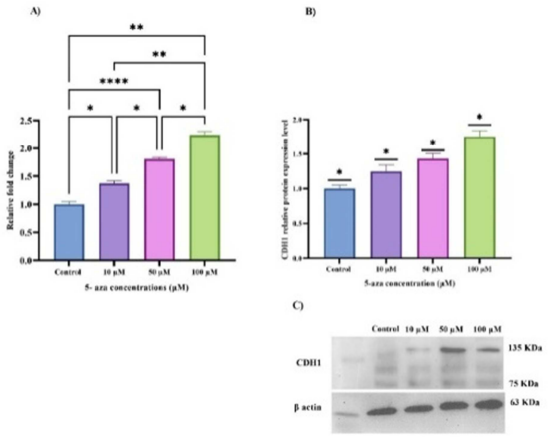

Figure 2: Relative *CDH1* gene and protein expression after 5-aza treatment. (A) mRNA levels were analysed by RT-qPCR and normalized to GAPDH. (B, C) Protein expression determined by Western blot and densitometry. Data are mean ± SD (n = 3 independent experiments). One-way ANOVA with Tukey's post hoc test was applied.Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Hypermethylation of the *CDH1* promoter

Previous studies have reported that the *CDH1* gene is hypermethylated in colorectal cancer (CRC). To evaluate the demethylating effect of 5-aza on the *CDH1* promoter, methylation-specific PCR (MSP-PCR) was performed to assess methylation patterns in LS513 cells following 48 h of 5-aza treatment. The results showed that treatment with 10 µM 5-aza did not alter the *CDH1* promoter methylation status compared with the control group. In contrast, partial demethylation was observed at 50 µM, while complete demethylation occurred at 100 µM, indicating a concentration-dependent effect (Figure 3).

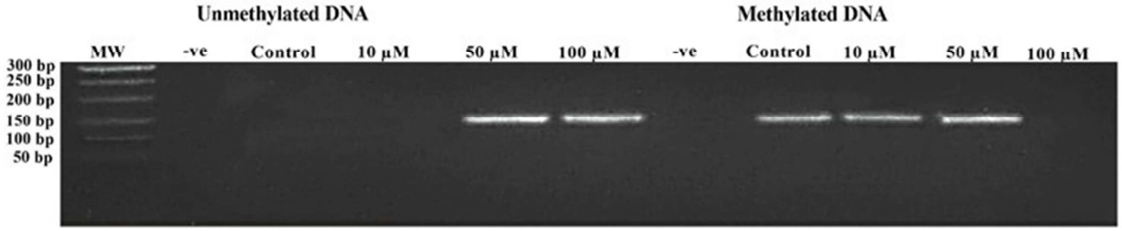

Figure 3: Demethylation of the *CDH1* promoter following 5-aza treatment. Hypermethylation of CpG dinucleotides in the *CDH1* promoter region was analysed using methylation-specific PCR (MSP-PCR). The results show both methylated (M) and unmethylated (U) DNA patterns in LS513 cells after treatment. *–ve*: negative control (PCR reaction without a DNA template); M: methylated; U: unmethylated bands.

Cell cycle analysis

To elucidate the potential effect of 5-aza on colorectal cancer (CRC) cell proliferation, LS513 cells were treated with 10, 50, and 100 µM of 5-aza, and DNA content was analyzed by flow cytometry after 48 h of treatment. Treatment with 10 µM 5-aza did not significantly alter the percentage of cells in the G_0/G_1 phase compared with the control. However, exposure to 50 and 100 µM 5-aza increased the proportion of LS513 cells in the G_2/M phase. Specifically, the percentage of cells in the G_2/M phase increased from 30.04 ± 1.42% in the control group to 35.15 ± 1.90% and 34.06 ± 2.42% at 50 and 100 µM, respectively (Figure 4C–E). Conversely, treatment with 50 and 100 µM 5-aza significantly reduced the proportion of cells in the G_0/G_1 phase, from 54.72 ± 2.16% in the control to 50.81 ± 3.26% and 47.63 ± 2.30%, respectively, and In the S phase, the percentage of cells slightly changed from 15.24 ± 1.65% in the control group to 14.10 ± 1.04% and 18.37 ± 0.41% at 50 µM and 100 µM 5-aza, respectively. Furthermore, 5-aza treatment at 10, 50, and 100 µM markedly increased the proportion of dead cells (pre-G_1 phase) from 1.56 ± 0.10% in the control to 2.02 ± 0.08%, 3.21 ± 0.11%, and 9.50 ± 0.19%, respectively (Figure 4F).

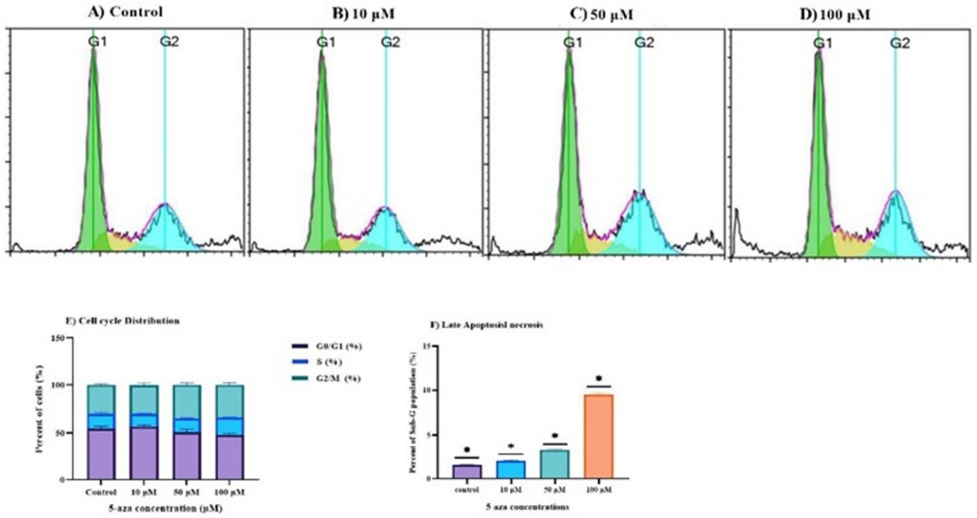

Figure 4: Distribution of LS513 cells across cell cycle phases after 48 h 5-aza exposure (10–100 µM). Data are mean ± SD (*n* = 3). Differences between treatment groups were analyzed by one-way ANOVA followed by Tukey's multiple comparison test.
p< 0.05 (*), *p* < *0.01* (**), *p* < *0.001* (***) vs. control; symbols (#) denote significance compared with 10 µM group.

5- aza induced autophagy progression

Autophagy is an essential form of programmed cell death and remains a major focus of cancer research. This study examined the effect of 5-aza at concentrations of 10, 50, and 100 µM on autophagy in LS513 cells using the Cyto-ID autophagy detection dye and flow cytometry. Treatment with 5-aza induced a dose-dependent increase in autophagic cell death, rising from 20.52 ± 3.9% in the control group to 23.52 ± 3.6%, 37.08 ± 5.07%, and 62.88 ± 2.10% at 10, 50, and 100 µM, respectively (Figure 5A–E).

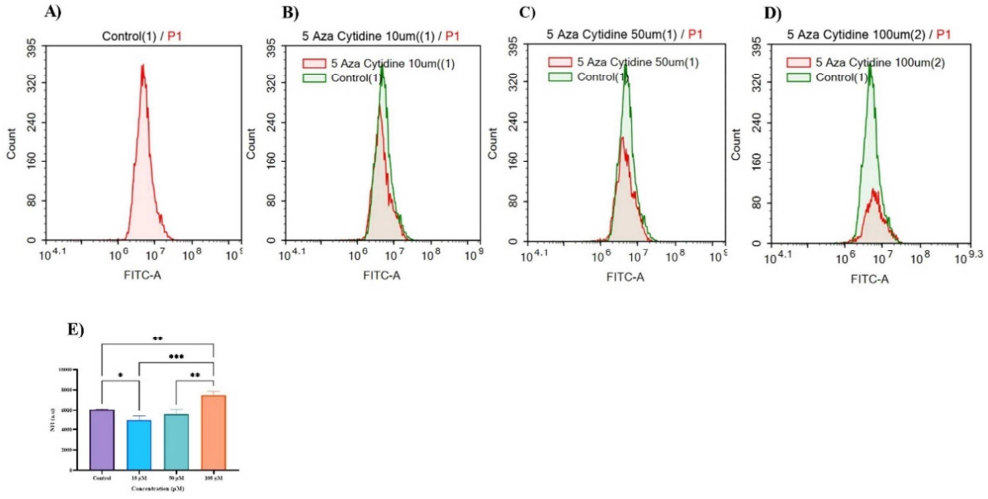

Figure 5: Evaluation of autophagic cell death in LS513 cells following 5-aza treatment. LS513 cells were treated with 5-aza at concentrations of 10, 50, and 100 µM for 48 h. Autophagy was assessed using the Cyto-ID autophagosome tracking dye and flow cytometry. Fluorescence intensity was quantified relative to the baseline fluorescence of control cells. Data are presented as mean ± SD from three independent experiments (*n = 3*). Statistical significance was conducted at *p*< 0.05.

4. Discussion

Colorectal cancer (CRC) continues to exhibit a high mortality rate worldwide. The five-year survival rate for patients with metastatic CRC remains below 10%, whereas early-stage diagnosis can increase survival rates to as high as 90% [18]. Despite advances in therapeutic strategies, current treatment approaches for colorectal tumors remain suboptimal, with high recurrence rates and the development of drug resistance [19]. Previous studies have reported that the *CDH1* gene is frequently hypermethylated in CRC [20–22]. Furthermore, 5-aza, a chemotherapeutic agent approved for several cancer types, has been shown to reduce promoter methylation of various genes, thereby restoring their expression [23-25]. In this study, the anticancer potential of 5-aza in CRC was investigated using the LS513 cell line. The results demonstrated that 5-aza inhibited cell growth in a dose-dependent manner. At lower concentrations, 5-aza had minimal effects on cell viability; however, at doses of 10 µM and above, a significant reduction in cell growth was observed. Notably, treatment with 50 and 100 µM 5-aza induced cell cycle arrest at the G_2/M phase. These findings are consistent with those reported by Sanaei *et al*. [26], who observed similar growth inhibition and antiproliferative effects in HCT-116 cells following 5-aza treatment. These resultscollectively suggest that 5-aza may serve as a promising therapeutic agent for CRC.To further elucidate the mechanism of action, this study examined the effect of 5-aza on *CDH1* gene expression and promoter methylation. The results revealed that treatment with 50 µM 5-aza partially demethylated the *CDH1* promoter, whereas 100 µM resulted in complete demethylation after 48 h of exposure. This alteration in methylation status led to a corresponding increase in CDH1 mRNA and protein expression, **indicating** that 5-aza **acts** in a concentration-dependent manner. These findings are in agreement with those of Li et al. [27], who reported that 5-aza treatment inhibited cell viability and enhanced *CDH1* expression. Similarly, Yamanaka et al. [28] found that *CDH1* hypermethylation was associated with reduced expression and increased invasiveness in malignant cells. Additionally, the present findings showed that 5-aza induced autophagy in LS513 cells, consistent with the observations of Filip et al. [29], who reported elevated autophagic activity in β-TC-6 cells treated with 5-aza. This suggests that 5-aza may exert its anticancer effects not only through demethylation and reactivation of tumor suppressor genes but also by triggering autophagy. Together, these results highlight the multifaceted role of 5-aza in CRC treatment and underscore the need for further investigation into its potential mechanisms and therapeutic applications.

5. Conclusion

This study demonstrates that 5-azacytidine effectively demethylates the hypermethylated *CDH1* promoter, leading to *CDH1* reactivation, G_2/M phase arrest, and induction of autophagy in colorectal cancer cells. These findings support the potential use of 5-azacytidine as an epigenetic therapeutic agent for colorectal cancer.Further studies using CDH1 knockdown or autophagy-inhibitor assays are required to verify whether CDH1 reactivation directly mediates the observed autophagic response.

6. Conflicts of interest

There is no conflict between the authors.

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8. Acknowledgments

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