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Research Article

Effects of 5-Fluorouracil on the Expression of Epigenetic Enzymes and Promoter Methylation of Selected Genes in Monolayer and Spheroid Cultures of Colorectal Cancer Cells

Maryam Niknam[1], Masoumeh Varedi[2], Mozhdeh Zamani[3], Pooneh Mokarram[3], Fakhraddin Naghibalhossaini[1,4*]

[1]*Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.*
[2]*Department of Physiology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.*
[3]*Autophagy Research Center, Department of Biochemistry, Shiraz University of Medical Sciences, Shiraz, Iran.*
[4]*Autoimmune Diseases Research Center, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.*

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Abstract

Background: Emerging evidence suggests that epigenetic mechanisms contribute to 5-fluorouracil (5-FU) resistance in colorectal cancer (CRC). However, there is limited research on the direct impact of 5-FU on epigenetic alterations in CRC. This study aimed to investigate how 5-FU treatment affects the expression of enzymes involved in epigenome regulation and promoter DNA methylation in human CRC cells.

Methods: The viability of CRC cell lines (SW48, HCT116, LS180, and HT29) was evaluated after 48 hours of 5-FU treatment using MTT assay in both monolayer and hanging drop spheroid cultures. The cells were treated with an IC_{20} concentration of 5-FU and then the relative expressions of histone deacetylases (*HDAC*) and DNA methyltransferas1 (*DNMT1*) in 5-FU-treated and untreated cells were measured by quantitative RT-PCR (qRT-PCR). The status of promoter methylation of selected genes was analyzed using the methylation-specific PCR (MSP) method.

Results: The 3D cultures of cells were more resistant to 5-FU than their 2D counterparts. The effect of 5-FU on *HDAC1* expression was greater in 3D cultures compared to 2D cultures. 5-FU downregulated *SIRT1* and *DNMT1* in 2D culture of HCT116 and SW48 and upregulated them in 3D cultures of HT29 and LS180 cells. In both monolayer and spheroid cultures, 5-FU downregulated *HDAC2* in HCT116, LS180, and HT29 and *HDAC4* in HCT116, LS180, and SW48 cells. 5-FU primarily changed promoter methylation in monolayer cultures.

Conclusion: The epigenetic response to 5-FU is cell line-specific and depends on the culture method. 5-FU modulates epigenome in CRC cells by regulating *DNMT1* and *HDAC* expressions. 3D cultures were found to be considerably more resistant to 5-FU-induced cytotoxicity and promoter DNA methylation changes than 2D cultures. 5-FU downregulated *HDAC* and *DNMT1*, particularly in the drug-sensitive cells, and increased the levels of DNMT1 in the drug-resistant cells.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, with 1.9 million incidences and 935,000 deaths estimated in 2020.[1] Since its discovery in 1957, 5-fluorouracil (5-FU) alone or in combination with other therapeutic agents has been used as standard adjuvant chemotherapy to treat CRC and other types of cancer patients.[2] The cytotoxicity of 5-FU is exerted through its metabolite, FdUTP which impairs DNA replication in dividing cells by irreversibly inhibiting the thymidylate synthase enzyme. Misincorporation of the fluorinated byproducts (FUTP and FdUTP) into RNA and DNA also disrupts functional RNA and protein synthesis causing tumor cell death. However, only 10–15 % of patients with advanced CRC respond effectively to 5-FU-based frontline chemotherapy and about 50% of CRC patients will eventually develop drug resistance and progress to metastatic CRC.[3]

Several intrinsic and acquired factors have been identified that contribute to the development of resistance to 5-FU chemotherapy, including mechanisms involved in drug transport, drug metabolism, and the repairing of drug-induced damage.[4] The metabolism of 5-FU is controlled by several enzymes including thymidylate synthase (TS),

*Corresponding Author: Fakhraddin Naghibalhossaini, E-mail: fakhraddin.naghibalhossaini@mail.mcgill.ca

dihydropyrimidine dehydrogenase (DPD), and thymidine phosphorylase (TP). Previous studies have demonstrated that the genetic variability of 5-FU metabolizing enzymes, multidrug transporters, and DNA repair significantly influence 5-FU chemoresistance and clinical outcomes in cancer patients.

Acquired drug resistance gradually occurs during treatment and reduces drug responsiveness after an initial positive response to the chemotherapy. Increasing evidence suggests that epigenetic changes may influence the development of resistance to conventional drugs such as 5-FU in CRC.[5] DNA methylation and modulation of chromatin structure through histone acetylation are two major epigenetic mechanisms that regulate gene expression. DNA methylation is catalyzed by three active DNA methyltransferases (DNMTs) (DNMT1, DNMT3A, and DNMT3B) that transfer the methyl group from S-adenosyl methionine (SAM) to the 5′ carbon of the cytosine bases at CpG sites of DNA. The histone acetylation state of chromatin is modified by the opposing activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes where they catalyze reversible acetyl transfer and removal on epsilon lysine residues on histone tails, respectively. Aberrant expression or mutations of genes encoding the components of the epigenetic machinery have been observed in various types of cancers.[6] The overexpression of HDAC genes in CRC patients has been linked to a higher mortality rate.[7,8] On the other hand, both in vitro and in vivo studies have shown that HDAC inhibitors or HDAC gene knockout effectively suppress the growth of CRC cells.[9,10] HDAC11 expression has been shown to suppress the invasion and metastasis of CRC cells by reducing the expression of matrix metalloproteinase3 (MMP3).[11] Both overexpression and loss of HDAC2 expression with implications in resistance to 5-FU chemotherapy have been reported in colon cancer cells.[12-14] A recent study of 1372 tumor samples by Zhou *et al.*[15] identified four distinct expression patterns of histone modifiers associated with different 5-FU sensitivities in colon cancer patients. In another study, Liu *et al.*[16] indicated that the decreased expression of acetyltransferase P300/CBP associated factor (PCAF), increases the resistance of CRC cells to 5-FU through the p53-mediated p21 expression mechanism.

Aberrant DNA methylation including gene-specific hypermethylation of CpG islands and global DNA hypomethylation, are early events in colorectal carcinogenesis.[17] Hypermethylation of CpG islands within the promoters of tumor suppressor genes contributes to the transcriptional silencing of these genes, ultimately leading to the development of cancer. Growing evidence suggests that aberrant DNA methylation may also contribute to the development of drug resistance in cancer cells through activating and silencing particular genes.[18,19] A genome-scale DNA methylation profiling of 43 non-recurrent and 5 recurrent tumor samples from CRC patients indicated that the DNA methylation levels in recurrent CRCs are higher than those in non-recurrent CRCs.[20] The methylation of the hMLH1 promoter, leading to the loss of DNA mismatch repair was also associated with 5-FU chemoresistance in CRC cells.[21] The findings from previous studies suggest that the tumor methylation status can influence prognosis and therapeutic response. The DNMT inhibitor 5-aza-deoxycytidine has shown a synergic effect on the cytotoxicity induced by 5-FU and reversed the drug resistance by increasing the expression of the 5-FU metabolizing enzyme, UMP kinase.[22,23] The hypermethylation of the protocadherin-17 promoter in CRC cells has been shown to increase 5-FU chemoresistance by inhibiting 5- FU-induced apoptosis and autophagy.[24] Previous in vitro studies have also indicated that 5-FU treatment may change DNA methylation status by modulating the expression of DNMTs in lung and breast cancer cells.[25,26]

It has been suggested that multicellular spheroid (3D) cultures better simulate the organization of cancer cells in a tumor than the conventional monolayer (2D) culture and could therefore be used to predict more accurately the chemotherapy response in preclinical studies.[27] While the association between epigenetic changes and drug resistance has been investigated in numerous research, only a few have examined the impact of 5-FU on the epigenome. In this study, we investigated how 5-FU affects the expression of genes involved in epigenetic regulation (*HDAC*s and *Dnmt1*) and the changes in promoter DNA methylation of selected genes in CRC cells grown in both 2D and 3D cultures.

Methods

Chemicals and reagents

Cell culture reagents were purchased from Gibco/Invitrogen (Paisley, UK). 5-FU (50 mg/ml) obtained from Pharma Resources GmbH, Alemania, stored at room temperature, and dissolved directly into the cell culture medium to desired final concentrations. All other chemicals and reagents used in this study, unless otherwise stated, were from Sigma-Aldrich (Munich, Germany).

Monolayer (2D) and multicellular spheroid (3D) cell cultures

Four human CRC cell lines (HCT116, HT29, LS180, and SW48) were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran). HCT116 and SW48 cells were maintained in RPMI 1640, and HT29 and LS180 cell lines in DMEM, both media supplemented with 10% fetal bovine serum (FBS), 2 mM Gln, 100 U/ml penicillin, and 100 µg/ml streptomycin, in a humidified 5% CO_2 atmosphere at 37°C. The cells were grown by culturing under standard 2D-monolayer or 3D-spheroid conditions, as described below.

The scaffold-free multicellular spheroids (MCS) were formed by the hanging drop method as previously described with minor modifications.[28] Briefly, a suspension of 7-8 $\times 10^3$ tumor cells per 30 µl media per droplet was

loaded on the lid of a 100 mm tissue culture Petri dish containing 10 ml of PBS, as a hydration chamber. After seeding, the cells were incubated at 37°C with 5% CO_2 in 95% humidity for 48 h to allow for gravity-enforced cell aggregation and the formation of multicellular spheroid. A phase contrast microscope was utilized to monitor the aggregate formation and the spheroid growth.

Cell cytotoxicity assay

To quantify the cytotoxicity of 5-FU in monolayer culture, CRC cells were seeded at a density of 7-8×10^3 cells per well containing 150 µl complete medium in 96-well plates. Twenty-four hours after seeding, the medium was replaced by a fresh complete medium
containing different doses of 5-FU (0, 5, 10, 25, 50, 100, and 150 µM) for 48 h. Cell viability was determined by MTT assay as previously described.[29] For each experiment, untreated cells were used as controls. To determine IC_{50} values, the dose–response curve was analyzed by the GraphPad PRISM software, version 5.00 (San Diego, CA). Each experimental treatment was performed in duplicate and repeated at least three times.

To evaluate the cytotoxicity of CRC cells grown in spheroids, the pre-formed hanging-drop spheroids were transferred to a 96-well plate and 5-FU was added to the spheroids at final concentrations ranging from 0 to 150 µM for 48 h. The viability of cells was measured by MTT assay as described above.

Drug treatment and DNA and RNA isolation

For monolayer culture, 25-35×10^4 cells were seeded in a 6 cm cell culture plate containing 5 ml complete growth medium for 24 h before drug treatment. For 3D culture, cancer cells (30×10^3) were seeded in non-adherent 6 cm bacterial Petri dishes in 5 ml complete media for 5 to 7 days to allow spheroid formation. To determine the effect of 5-FU on DNA methylation and the expression of genes, both monolayer and spheroid cultures were treated with 5-FU at a minimally toxic concentration of IC_{20} (Table 1) for 48 hours. Total RNA was extracted from 5-FU-treated and untreated control cells of monolayer and spheroid cultures using a BIOZOL RNA isolation kit (Bioflux-Bioer, China), following the manufacturer's instructions. The integrity of the purified RNA samples was verified by electrophoresis on 1.5% denaturing agarose gels containing 2% formaldehyde.

Genomic DNA was extracted from 5-FU-treated and untreated cancer cells cultured as monolayer or spheroid by the standard method of proteinase K digestion followed by phenol-chloroform extraction.

Quantitative RT-PCR (qRT-PCR)

The relative gene expression levels of target genes (*HDAC1, 2, 3, 4, SIRT1, and DNMT1*) in 5-FU-treated and untreated cancer cell lines, were determined by real-time RT-PCR assay as described previously.[30] Briefly, the complementary DNA (cDNA) was synthesized by reverse transcription (RT) of 2 µg total RNA using oligodT primers and M-MuLV reverse transcriptase enzyme in 20 µl reaction volume following the protocol provided by the enzyme supplier (Cinagene, Iran). cDNA served as the template for PCR amplification using gene-specific primer sets (Table 2) and the identities of the PCR amplicons were verified by standard agarose gel electrophoresis. Real-time PCR quantification of each gene was performed on 1 µl of cDNA template in a 25 µl reaction mixtures using SYBR Green master mix (Ampliqon, Danmark) in a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems, USA). The amplification of genes was carried out in triplicate with a precycling heat activation at 95 °C for 10 min, followed by 40 cycles of heat denaturation at 95 °C for 15 s, annealing at the specified temperature (Table 2) for 30 s, extension at 72 °C for 30 s, and a last extension at 72 °C for 10 min. The expression level of target genes was normalized to the expression of *GAPDH* internal control using the $2^{-\Delta\Delta CT}$ formula.[31]

Table 1. Growth inhibitory effect of 5-FU against human CRC cell lines after 48 h treatment.

CRC Cell line	5-FU IC_5 (µM)	P	5-FU IC_{10} (µM)	P	5-FU IC_{20} (µM)	P	5-FU IC_{50} (µM)	P
HCT116								
Monolayer	0.239±0.078	0.0001	0.635±0.161	<0.0001	2.070±0.369	<0.0001	19.867±1.583	<0.0001
Spheroid	1.484±0.170		2.902±0.264		6.535±0.426		30.889±1.317	
SW48								
Monolayer	0.011±0.005	0.007	0.056±0.019	0.0003	0.421±0.094	0.0007	19.847±2.01	<0.0001
Spheroid	0.027±0.013		0.134±0.046		0.946±0.210		39.718±4.591	
HT29								
Monolayer	0.046±0.025	0.004	0.198±0.082	0.0004	1.161±0.303	<0.0001	34.18±4.886	<0.0001
Spheroid	0.266±0.116		0.891±0.287		3.841±0.813		62.899±7.526	
LS180								
Monolayer	0.293±0.094	0.0002	0.943±0.226	<0.0001	3.882±0.611	<0.0001	58.223±5.695	<0.0001
Spheroid	1.245±0.394		3.168±0.751		9.822±1.584		85.556±16.839	

Results were presented as mean ± SD of two independent experiments in triplicate. Statistical difference was analyzed by Unpaired T-Test.

Table 2. Primers' sequence and annealing temperature used for quantitative RT-PCR.

Gene	Forward Primer	Reverse Primer	Annealing T (ºC)	Product size (bp)
DNMT1	5'-TACCTGGACGACCCTGACCTC-3'	5'-CGTTGGCATCAAAGATGGACA-3'	60	103
GAPDH	5'-CGACCACTTTGTCAAGCTCA-3'	5'-AGGGGTCTACATGGCAACTG-3'	60	232
HDAC1	5′-GGAAATCTATCGCCCTCACA-3'	5′-AACAGGCCATCGAATACTGG-3′	56	168
HDAC2	5′-TAAATCCAAGGACAACAGTGG-3′	5′-GGTGAGACTGTCAAATTCAGG-3′	56	89
HDAC3	5′-TAGACAAGGACTGAGATTGCC-3′	5′-GTGTTAGGGAGCCAGAGCC-3′	56	120
HDAC4	5′-GGTTTATTCTGATTGAGAACTGG-3′	5′-ATTGTAAACCACAGTGCTCGC-3′	56	146
SIRT1	5′-TGCGGGAATCCAAAGGATAAT-TCAGTGTC-3′	5′-CTTCATCTTTGTCATACTTCATG-GCTCTATG-3′	60	200

Table 3. Primers' sequence and the annealing temperature used for Methylation Specific PCR.

Gene	Forward Primer	Reverse Primer	Annealing T (ºC)	Product size (bp)
hMLH1	U:5'-TTTTGATGTAGATGTTTTATTAGG-GTTGT-3'	5'-ACCACCTCATCATAACTACCCAC A-3'	58	118
	M: 5'- ACGTAGACGTTTTATTAGGGTCGC-3'	5'- CCTCATCGTAACTACCCGCG-3'		124
MMP2	U:5'-GTGGTTATATGTATTGAGTTAGT-GATTTTTGGGTG-3'	5'- AAAAAACAAAACACCCTCAAAAAAC-CCATAAACA-3'	60	96
	M: 5'- TATCGAGTTAGCGATTTTCGGGC-3'	5'- CGCCCTCAAAAAACCCGTAAACG-3'		96
p16	U: 5'- TTATTAGAGGGTGGGGTGGATTGT-3'	5'- CAACCCCAAACCACAACCATAA-3'	60	151
	M: 5'- TTATTAGAGGGTGGGGCGGATCGC-3'	5'- GACCCCGAACCGCGACCGTAA-3'		149
p21	U: 5'- TTTTTGTAGTATGTGAGGTTTTGG-3'	5'- AACACAACTCAACACAACCCTA-3'	54	200
	M: 5'- TGTAGTACGCGAGGTTTCG-3'	5'- TCAACTAACGCAACTCAACG-3'		202
hTERT	U: 5´-AGTTTTGGTTTTGGTTATTTTTGT-3´	5´-AACGTAACCAACGACAACACCT-3´	58	132
	M: 5´-AGTTTTGGTTTCGGTTATTTTCGC-3´	5´-AACGTAACCAACGACAACACC-3´		122

U: Unmethylated, M: Methylated

Analysis of the gene promoter methylation by methylation-specific PCR (MSP)

The status of the promoter methylation of 5 candidate tumor-associated genes (*hMLH1*, *hTERT*, *MMP2*, *p16INK4a*, and *p21*) in 5-FU treated and untreated control cells were determined by the MSP method as described previously.[32,33] Briefly, one microgram of the extracted genomic DNA from CRC cells was treated with sodium bisulfite and then subjected to PCR amplification using two primer sets (Table 3) specific for both methylated and unmethylated CpG sites in the promoter region of genes. The MSP products were analyzed by 1.5% agarose gel electrophoresis and visualized under UV illumination.

Statistical analysis

Statistical analyses were performed using the SPSS version 18 (SPSS Inc., Chicago, IL) software package. Data are presented as mean ± standard deviation (SD). The difference between the two groups was evaluated by an unpaired Student's t-test. A P-value less than 0.05 ($p < 0.05$) was considered statistically significant.

Results

In the present study, we investigated the effect of 5-FU on the expression of enzymes involved in the epigenetic process and promoter methylation change in human CRC cell lines. Since epigenetic changes depend on cell division, we used the minimally cytotoxic concentration of 5-FU (IC_{20}) to detect the treatment-related changes, but not general non-specific chemically induced cytotoxic effects.[34]

Chemosensitivity of CRC cell lines to 5-FU in 2D and 3D cultures

We first determined the dose-response curves for 5-FU cytotoxicity in 4 CRC cell lines grown as 2D and 3D cultures. The cells were treated with different concentrations of 5-FU for 48 h and the response to treatment was analyzed by MTT viability assay (Figure 1). In the monolayer culture, SW48 (IC_{50}, 19.85 µM) and HCT116 (IC_{50}, 19.87 µM) were the most sensitive cell lines, followed by HT29 (IC_{50}, 34.18 µM), whereas LS180 cell showed the highest resistance to 5-FU treatment (IC_{50}, 58.22 µM) (Table 1).

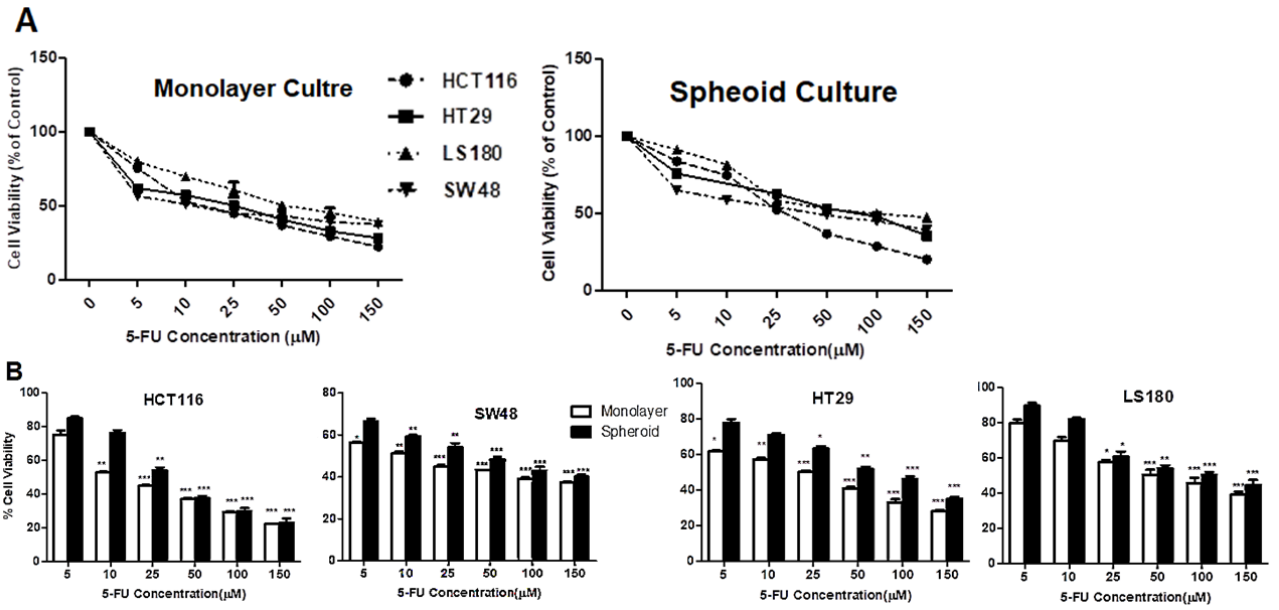

Figure 1. A. The dose-response curve between 5-FU concentration and cell viability of 4 CRC cell lines in monolayer and hanging-drop MCS cultures. Cells were treated with different concentrations of 5-FU for 48 h. Cell viability was determined using the MTT assay. The results are expressed as the percentage of the viability of control cells. **B.** Bar graph representation of the sensitivity of CRC cell lines to growth inhibition by 5-FU. The viability of cells was shown relative to the untreated control cells. Data are the means ± SD of three independent experiments, each done in duplicate. *p < 0·05, ** p <0.01, and ***p<0.001 as analyzed by unpaired T-Test.

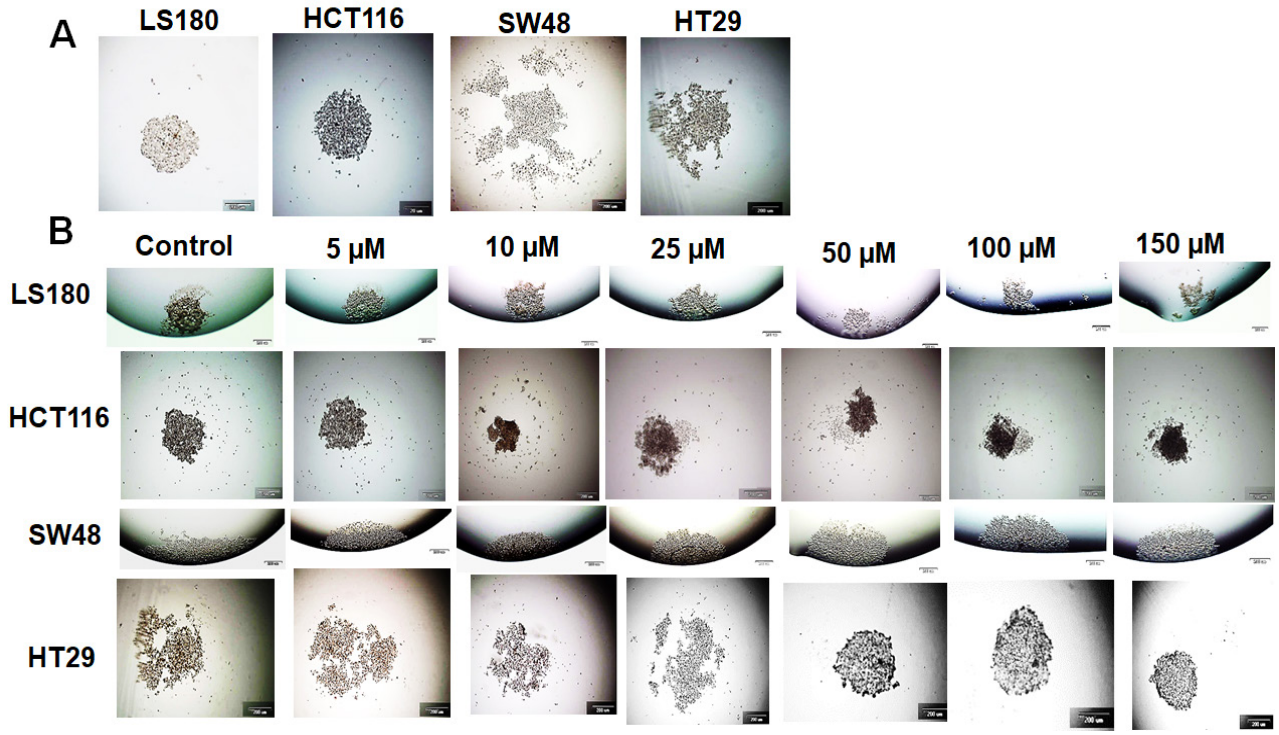

Figure 2. Micrograph of hanging-drop MCS of 4 CRC cell lines. **A.** The morphology of spheroids 24 hours post-seeding of 7000-8000 cells/hanging drop. **B.** The diameter and morphology of MCS 48 hours after exposure to the specified concentrations of 5-FU. Magnification 10x; Scale bar represents 200 μm.

Because 3D culture is considered a better preclinical cancer model, the cytotoxicity effect of 5-FU was also examined in 3D spheroids formed by the hanging drop method. An initial suspension of 7-8 $\times 10^3$ tumor cells per hanging drop allowed for the development of large multicellular aggregates within 24 h (Figure 2A). The MCS cultures were exposed to 5-FU for 48 h and the cell viability was determined as described above. The MCS cultures of CRC cell lines (Figure 2B) showed more resistance to the cytotoxic effect of 5-FU than their monolayer counterparts,

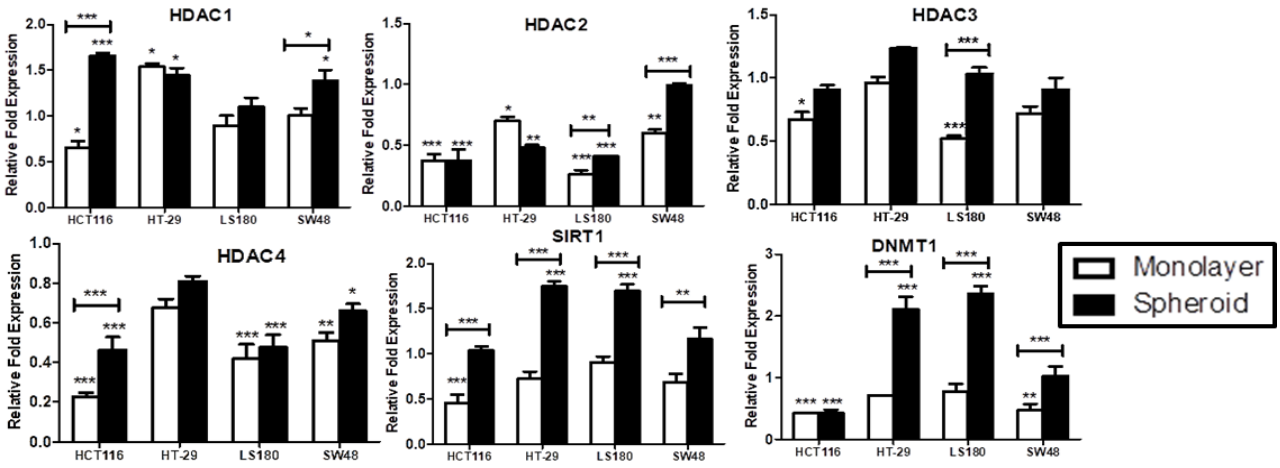

Figure 3. The effects of 5-FU on the expression of HDAC and DNMT genes in CRC cells. The monolayer and spheroid cultures of 4 CRC cell lines were treated with IC20 concentration of 5-FU for 48 h. The relative transcription level of genes was measured by RT-qPCR and normalized to the GAPDH expression level. The control untreated cells were used as a reference and their expression level was set to 1.0 and the expressions in all other cells were expressed relative to the control. The data are means ± SD of two independent assays in triplicate. *p < 0·05, **p < 0.01, and ***p<0.001, as analyzed by Unpaired T-Test.

Table 4. A summary of the change in the expression of the epigenome modifying genes in CRC cell lines treated with 5-FU as compared to the untreated control cells.

	HDAC1		HDAC2		HDAC3		HDAC4		SIRT1		DNMT1	
	S	M	S	M	S	M	S	M	S	M	S	M
HCT116	↑	↓	↓	↓	—	↓	↓	↓	—	↓	↓	↓
HT29	↑	↑	↓	↓	—	—	—	—	↑	—	↑	—
LS180	—	—	↓	↓	—	↓	↓	↓	↑	—	↑	—
SW48	↑	—	—	↓	—	—	↓	↓	—	—	—	↓

↓:Decreased,↑:Increased, —: No change, M: Monolayer culture, S: Spheroid culture

as reflected by their higher IC_{50} values (Table 2). For MCS cultures, the IC_{50} value was 85.56±16.83 µM for LS180, 62.9±7.53 µM for HT29, 39.72±4.59 µM for SW48, and 30.89±1.32 µM for HCT116 cells. As shown in Figure 1B, 5-FU in the concentration range of 5-25 µM, significantly decreased the viability of four CRC cell lines in both monolayer and spheroid cultures; however, the percentage of dead cells was markedly higher in 2D cultures in comparison to their 3D counterparts.

Effects of 5-FU on the expression of histone deacetylases

Dysregulation of HDAC genes in human CRC as well as synergistic antitumor effects of HDAC inhibitors with 5-FU have been reported by several studies.[35-37] To investigate the effect of 5-FU on the expression of HDAC genes, the monolayer and MCS cultures of four human CRC cell lines were treated with minimally cytotoxic concentration (IC_{20}) of 5-FU for 48 h followed by measuring the expression level of five *HDAC* genes (*HDAC1-4* and *SIRT1*) and *Dnmt1* by quantitative real-time RT-PCR analysis.

Effects of 5-FU on the expression of HDAC1

The quantitative RT-PCR assay indicated that the 5-FU effect on *HDAC1* expression level was higher in 3D culture than in 2D monolayer culture. As shown in Figure 3 and Table 4, *HDAC1* expression significantly increased by 5-FU in MCS cultures of HCT116 (65.45%, p<0.001), HT29 (44.28%, p=0.02), and SW48 (38.35%, p<0.05) cells, as compared with their respective untreated controls (p<0.05). The relative gene expression level of *HDAC1* expression in the 5-FU treated 2D culture of HT29 cells was also increased (53.7%, p = 0.01) compared to the untreated cells. 5-FU did not change the level of *HDAC1* transcript in 2D-cultures of SW48 and LS180 cells, but significantly reduced its expression in HCT116 cells (35.2%, P<0.05) (Figure 3 and Table 4).

Effects of 5-FU on the expression of HDAC2

As shown in Figure 3 and Table 4, the expression of *HDAC2* was inhibited to different extents by 5-FU treatment in both 2D and 3D cultures of CRC cells. The incubation of monolayer and spheroid cultures of HCT116 with 5-FU resulted in a 62.7% decreased expression of *HDAC2* (p<0.01). 5-FU also significantly inhibited *HDAC2* expression in monolayer and spheroid cultures of HT29 (30.4% and 54.5%, respectively) and LS180 (74.35%, and 59.375%, respectively) (p<0.05). In the monolayer culture of SW48, 5-FU downregulated *HDAC2* transcript by 39.8% (p=0.02), but did not alter the expression in the 3D culture of this cell line.

Effects of 5-FU on the expression of HDAC3 and HDAC4

5-FU treatment caused significant reduction of *HDAC3* transcript levels only in the monolayer cultures of HCT116

(45.6%, p<0.01) and LS180 (48.33%, p<0.01) cells. There was no significant change in the expression levels of *HDAC3* in either 2D or 3D cultures of other cell lines (Figure 3 and Table 4).

HDAC4 transcript level was significantly reduced in both monolayer and spheroid cultures of 5-FU treated HCT116 (77.35%, and 53.65%, respectively), LS180 (60.75%, and 52.525%, respectively), and SW48 cells (49.1%, and 34.15%, respectively) (p<0.05). A non-significant reduction of the *HDAC4* transcript level was also detected in 5-FU treated monolayer (33.3%, p = 0.081) and spheroid (19.125%, p = 0.24) cultures of the HT29 cells (Figure 3 and Table 4).

Effects of 5-FU on the expression of SIRT1

While the incubation of spheroid cultures of HT29 and LS180 cells with 5-FU resulted in the upregulation of *Sirt1* transcript (74.5% and 69.7%, respectively) (p<0.01), a very slight reduction of the expression was observed in 5-FU treated monolayer culture of these cells, as compared with their respective untreated controls (Figure 3 and Table 4). 5-FU treatment also downregulated *Sirt1* expression in the monolayer (54.63%, p=0.001), but not in the spheroid culture of HCT116 cells. A small but non-significant reduction in the expression of *Sirt1* was also found after the 5-FU treatment of SW48 monolayer culture (31.75%, p=0.06).

Effects of 5-FU on the expression of DNMT1 gene

5-FU treatment of 4 CRC cell lines increased *DNMT1* transcript levels in MCS cultures of HT29 and LS180 cell lines (2.12- and 2.36-fold, respectively), as compared to their respective non-treated control cells (p<0.01) (Figure 3 and Table 4). 5-FU treatment downregulated the expression of *DNMT1* in both monolayer (57.6%, p < 0.01) and spheoid (57.9 %, p<0.01) cultures of HCT116 and monolayer culture of SW48 (52.3 %, p<0.01). A non-significant reduction of *DNMT1* transcript levels was also observed in monolayer cultures of HT29 and LS180 cells (p>0.05).

Effects of 5-FU on the promoter DNA methylation

To evaluate DNA methylation changes in response to 5-FU treatment, we determined the promoter methylation status of five cancer-related genes in pre- and post-treated CRC cell lines by MSP analysis, as described in the "Methods" section. A total of 16 out of 40 genes (40%) analyzed for the promoter methylation, were fully methylated and 11 genes (27.5%) were hemimethylated in the untreated control cells in either 2D monolayer or 3D spheroids cultures (Figure 4 and Table 5). Five differentially methylated genes were identified between the untreated monolayer and spheroid cultures in LS180 and HT29 cells (*p16*, *hTERT*, and *hMLH1* in LS180; hTERT and *MMP2* in HT29). As shown in Figure 4 and Table 5, the 5-FU effect on the promoter methylation changes was more evident in monolayer than in spheroid cultures. 5-FU treatment converted the fully methylated promoter of *p16* and *hMLH1* into hemimethylated form in the 2D culture of SW48 and LS180 cells, respectively. The fully methylated promoter of *hTERT* was also changed into a hemimethylated form by 5-FU in the 2D culture of HCT116 and SW48 cells. The unmethylated promoters of *p21* and *MMP2* were converted into hemimethylated forms by 5-FU in monolayer cultures of SW48 and LS180 cells, respectively. 5-FU treatment also resulted in the

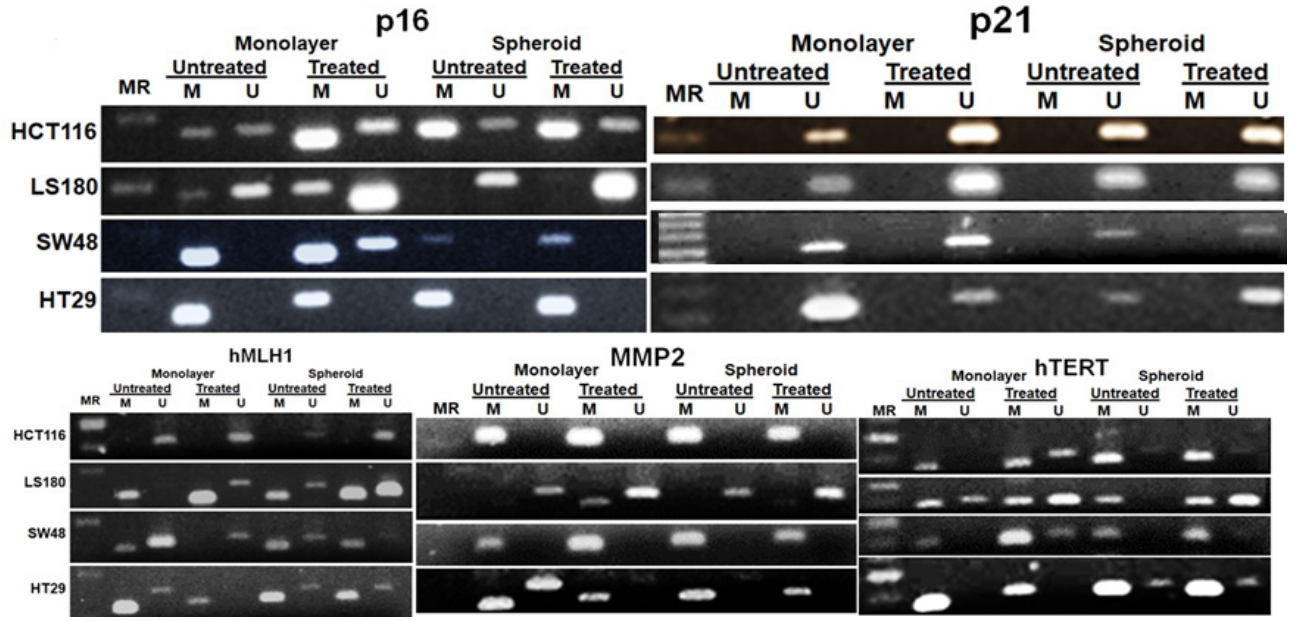

Figure 4. Methylation analysis of promoter region of 5 tumor-related candidate genes before and after 5-FU treatment of CRC cells. The monolayer and spheroid cultures of cancer cells were treated with IC_{20} concentration of 5-FU for 48 h and genomic DNA was extracted and analyzed for the status of the promoter methylation by MSP assay, as described in the Materials and Methods. M: Methylated promoter; U: unmethylated promoter; MR: DNA size marker. The genes containing both the methylated (M) and the unmethylated (U) promoters are hemimethylated (one of two complementary strands of DNA in the promoter region is methylated and the other one is unmethylated).

Table 5. The schematic representation of 5-FU effects on the promoter DNA methylation in CRC cells.

	Monolayer culture		Spheroid culture	
Cell line	**Untreated**	**Treated**	**Untreated**	**Treated**
		Gene: *p16*		
HCT116	●○	●○	●○	●○
LS180	●○	●○	○	○
SW48	●	●○	●	●
HT29	●	●	●	●
		Gene: *p21*		
HCT116	○	○	○	○
LS180	○	○	○	○
SW48	○	○	○	○
HT29	○	○	○	○
		Gene: *hMLH1*		
HCT116	○	○	○	○
LS180	●	●○	●○	●○
SW48	●○	○	●○	●○
HT29	●○	●	●○	●○
		Gene: *MMP2*		
HCT116	●	●	●	●
LS180	○	●○	○	○
SW48	●	●	●	●
HT29	●○	●	●	●
		Gene: *hTERT*		
HCT116	●	●○	●	●
LS180	●○	●○	●	●○
SW48	●	●○	●	●
HT29	●	●	●○	●○

(●) methylated promoter; (○): unmethylated promoter. The genes containing both the methylated (●) and the unmethylated (○) promoters are hemimethylated (one of two complementary strands of DNA in the promoter region is methylated and the other one is unmethylated).

conversion of the hemimethylated promoter of MMP2 into a fully methylated form in the monolayer culture of the HT29 cell line. Among the spheroid cultures, hTERT in LS180 cells was the only differentially methylated gene detected between the 5-FU treated and untreated cells, so the methylated promoter of hTERT was converted into hemimethylated form by 5-FU treatment (Figure 4 and Table 5).

Discussion

In vitro drug testing is crucial for modern precision medicine in oncology and the majority of in vitro drug testing has been performed using 2D monolayer cultures of tumor cells. However, increasing evidence suggests that 3D spheroid cultures are superior for in vitro drug screening due to their better simulation of tumor architecture in vivo. The present study aimed to investigate the effects of 5-FU on the expression of genes encoding regulators of epigenome and on the promoter methylation of selected tumor-related genes in colorectal cancer cells. To determine the minimally cytotoxic concentration of 5-FU, the viability of cells was measured in 2D and 3D cultures by MTT assay. As shown in Figure 1, the MSC cultures were more resistant to 5-FU than the monolayer cultures. IC_{50} values for the spheroid cultures were 1.5 – 2.1 fold higher than the corresponding values for the monolayer culture. Consistent with our data, several studies noted that CRC cells in MCS culture are more resistant to anticancer drugs than the same cells in monolayer cultures.[29,38,39] In our study, LS180 had the highest IC_{50} value and the rank order for IC_{50} value was the same for these cells in both 2D and 3D cultures (LS180 > HT29 > SW48 > HCT116) (Table 1). In agreement with our results, previous studies reported a similar rank order of resistance to 5-FU for 3 of these CRC cell lines, ranging from high (HT29), intermediate (SW48), and low (HCT116).[22,40] Several factors have been suggested to play roles in increasing cell resistance to 5-FU in MCS compared to monolayer culture including decreased drug penetration inside the cell mass of spheroids and altered gene expression because of changes in cell-cell and cell-matrix interactions within the spheroid.[41] Spheroid cohesion has been also suggested to influence the resistance of aggregated cells to the cytotoxicity of 5-FU treatment.[40] It has been shown that drugs could not diffuse equally to all regions of spheroids and compact spheroids are more resistant to drugs than looser spheroids.[38] HT29 cell line enriched in E-Catherin/catenin cell-to-cell junction has been shown to form a high cohesive aggregate and is much more resistant to 5-FU cytotoxicity than HCT116 and SW480 metastatic cell lines.[40,42] In our previous study, we found a positive correlation between the carcinoembryonic antigen (CEA), an intercellular adhesion molecule, expression level, and resistance to 5-FU in CRC cells. We also showed a high expression level of CEA protein in the LS180 cell line.[28,43] These findings accord with the result of the present study, as LS180 cell line demonstrated the

highest level of 5-FU resistance among the four CRC cell lines tested, in both monolayer and spheroid cultures.

DNA methylation and histone modifications play key roles in the proliferation and development of colonic epithelial cells.[10,34,44] HDACs are frequently dysregulated in CRC and both the upregulation and loss of expression have been reported. Although numerous studies have demonstrated the association between epigenetic changes and 5-FU chemoresistance, limited studies investigated the impact of 5-FU on the epigenome-modifying enzymes in CRC cells.[45,46]

In this study, we investigated the influence of 5-FU on the expression of HDACs and DNMT1 in 2D and 3D cultures of 4 human CRC cell lines. The effects were gene- and cell-type-specific, 5-FU treatment coordinately suppressed the expressions of HDAC2 in the monolayer and spheroid cultures of HCT116, HT29, and LS180 cells (Figure 3B and Table 4). The HDAC4 expression was also coordinately suppressed by 5-FU treatment in 2D and 3D cultures of HCT116, LS180, and SW48. In the monolayer culture, 5-FU downregulated HDAC1 and HDAC3 in HCT116, HDAC2 in SW48, and HDAC3 expression in LS180 cells.

5-FU has been shown to promote chemoresistance by inducing global histone deacetylation and the degradation of p300/CBP in some CRC cell lines.[45] Based on clinical data from 262 CRC patients, it has been suggested that the low expression of histone acetyltransferases p300/CBP in tumor tissues correlates with poor response to 5-FU-based chemotherapy and a lower disease-free survival rate.[45] The decreased expression of P300/CBP associated factor (PCAF) was also demonstrated to increase the resistance of CRC cells to 5-FU cytotoxicity through p53-dependent downregulation of the p21 mechanism.[16] HDAC2 expression has been also shown to correlate with the response of colon cancer cells to 5-FU. Kiweler and colleagues reported that HDAC2-negative RKO CRC cells have decreased sensitivity to 5-FU-induced apoptosis compared to HDAC2-expressing RKO cells.[12] In the present study, 5-FU exposure induced HDAC1 expression in monolayer and spheroid cultures of HT29 and in spheroid cultures of HCT116 and SW48 cells. Preclinical studies have shown that the combination of HDAC inhibitors (HDACi) with 5-FU can overcome resistance to 5-FU treatment in CRC cells.[47,48] This synergistic antitumor effect of HDACi was suggested to be mediated through the downregulation of thymidylate synthase. As shown in Figure 3, 5-FU did not have a significant effect on the transcript levels of HDAC1 in LS180 cells, HDAC3 in HT29 and SW48 cells, HDAC4 in HT29 cells, and SIRT1 in SW48 cells when grown as monolayers or spheroids. However, 5-FU treatment upregulated SIRT1 and DNMT1 expressions in the spheroid cultures of HT29 and LS180 cells and downregulated their expressions in the monolayer cultures of HCT116 and SW48 cells. In this study, the expression of DNMT1 was coordinately suppressed by 5-FU in 2D and 3D cultures of HCT116 cells. Cao and colleagues have also reported that 5-FU downregulates the expression of DNMT1 and DNMT3b in A549 lung adenoma-carcinoma cells. The downregulations caused epigenetic enrichment of cancer stem cells, resulting in higher drug resistance.[49]

Sirtuins are a family of 7 NAD-dependent histone deacetylases (SIRT1-7) that also deacetylate nonhistone proteins. For example, SIRT1 also deacetylates p53 and HINT1 proteins and may function as a tumor suppressor in CRC cells.[50,51] Tang *et al.*[46] suggested that 5-FU exposure promotes SIRT7 degradation through the Tat-binding Protein 1 (TBP1) proteasome pathway resulting in increased radiosensitivity of CRC cells.They indicated that decreasing SIRT7 levels by 5-FU sensitizes tumors in NOD-SCID mice to irradiation and improves the therapeutic efficacy of 5-FU and radiation in rectal cancer cells.

Increasing evidence supports the notion that epigenetic mechanisms play roles in the development of 5-FU resistance in CRC.[21] The promoter DNA methylation of the hMLH1 mismatch repair gene was shown to correlate with the 5-FU resistance in CRC cells.[15] A previous study on a panel of 77 CRC cell lines has also indicated the association between mismatch repair deficiency and 5-FU chemoresistance. In this study, we compared the promoter methylation of 5 tumor-related genes in 2D and 3D cultures of cancer cells. We also investigated the effect of 5-FU on DNA methylation changes in both 2D and 3D cultures. Figure 4 and Table 5 indicate the difference in the promoter methylation of the studied genes in the monolayer versus spheroid culture. Certain genes showed a higher sensitivity to the culture method in terms of changes in DNA methylation. Differential promoter methylation between monolayer and spheroid cultures was observed for *p16*, *hMLH1*, and *hTERT* genes in LS180, and *MMP2* and *hTERT* genes in HT29 cell lines. A previous study by DesRochers *et al.*[52] identified significant differences in DNA methylation and gene expression between 2D and 3D culture systems of squamous cell carcinoma. Wang and colleagues[53] compared DNA methylation differences among 2D, 3D, and mouse orthotopic transplantation cultures in the DLD-1 CRC cell line. They concluded that the culture method has no influence on DNA methylation changes in the DLD-1 cell line.In the present study, the influence of the cell culture method on promoter DNA methylation was gene- and cell-line dependent. Our results indicated that monolayer cultures were more sensitive to the 5-FU-induced DNA methylation changes than spheroid cultures. In 8 out of 9 cases, the change in promoter methylation induced by 5-FU was found in the monolayer cultures. There was only one methylation change induced by 5-FU among the spheroid cultures, which was the demethylation of the hTERT promoter in the 3D culture of LS180 cells (Figure 4 and Table 5). In monolayer cultures, 5-FU induced demethylation of *p16*, *hMLH1*, and *hTERT* genes in the drug-sensitive SW48 cells, demethylation of *hMLH1* in LS180 and *hTERT* in HCT116, and hypermethylation of *MMP2* and *hMLH1* in HT29 and *MMP2* promoter in LS180 cells. A biphasic response was described for drug-induced DNA methylation changes

in cancer cells: hypermethylation occurring at high drug concentration (killing more than 90% of exposed cells) and hypomethylation occurring at low to moderately cytotoxic drug concentration (killing less than 50% of cells).[54] These findings align with our observation of a decrease in promoter methylation in CRC cells following treatment with a low dose (IC_{20}) of 5-FU (Figure 4).

DNA methylation is performed by three DNMT enzymes (DNMT1, DNMT3A, and DNMT3B). DNMT3A and 3B mainly control global de novo methylation during embryonic development, but DNMT1 is involved in preserving methylation patterns in proliferating cells. Several studies reported drug-induced DNA methylation changes in human tumor cells.[24,25,55] 5-FU has been reported to change DNA methylation levels in human lung and breast cancer cells by decreasing the expression of DNMT enzymes. Exposure to 5-FU and 5-fluorodeoxyuridine has been shown to induce DNA hypermethylation in human lung adenocarcinoma HTB-54 and human rhabdomyosarcoma CCl-136 cells.[55] A previous genome-wide methylation study on clinical specimens showed that recurrent CRCs exhibit higher methylation levels than non-recurrent CRCs.[19] The same study indicated that 5-azadC treatment successfully sensitizes cancer cells to 5-FU treatment. Drug-induced aberrant promoter hypermethylation has been proposed as a mechanism that promotes drug resistance by inactivating genes whose products are required for drug cytotoxicity.[54] These genes are primarily involved in pyrimidine metabolism, drug metabolism-cytochrome P450, epidermal growth factor receptor signaling, and p53 signaling pathways.[54] In this study, we found that 5-FU decreased the expression of DNMT1 in 5-FU sensitive cells (SW48 and HCT116), but increased in 5-FU resistant cells (LS180 and HT29), suggesting a positive link between 5-FU-induced DNMT expression and 5-FU chemoresistance (Figure 3). A positive correlation between the expression levels of DNMT enzymes and global DNA methylation levels in CRC cells has been reported.[56] A subset of CRC tumors exhibits consistent and frequent hypermethylation of multiple CpG islands that are unmethylated in other CRC tumors or normal tissues. This phenotype is referred to as the CpG island methylator phenotype (CIMP).[57] It has been suggested that CRC patients with CIMP tumors may not benefit from undergoing 5-FU-based chemotherapy treatment.[58,59]

Conclusion

We found that CRC cells in 3D cultures are more resistant to 5-FU than those in 2D cultures. Various CRC cell lines showed varying levels of sensitivity to 5-FU. HCT116 and SW48 exhibited high sensitivity, HT29 showed an intermediate sensitivity, and LS180 cells had low sensitivity. 5-FU treatment had profound effects on the expression of genes that regulate the epigenome. 5-FU reduced the expression of HDAC genes in the majority of cells, particularly in the 5-FU-sensitive HCT116 cell line. 5-FU increased the DNMT1 expression in the drug-resistant cells but decreased it in the sensitive cell lines. 5-FU treatment primarily altered promoter DNA methylation in CRC cells grown in monolayer culture, as compared to spheroid culture. Additional studies are needed to determine the precise pharmacoepigenetic mechanism behind the acquired resistance to 5-FU-based chemotherapy.

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Author Contributions

Maryam Niknam: Investigation. Masoumeh Varedi: Methodology, Supervision. Mozhdeh Zamani: Formal analysis, Pooneh Mokarram: Formal analysis, and Fakhraddin Naghibalhossaini: Conceptualization.

Conflict of Interest

The authors report no conflicts of interest.

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