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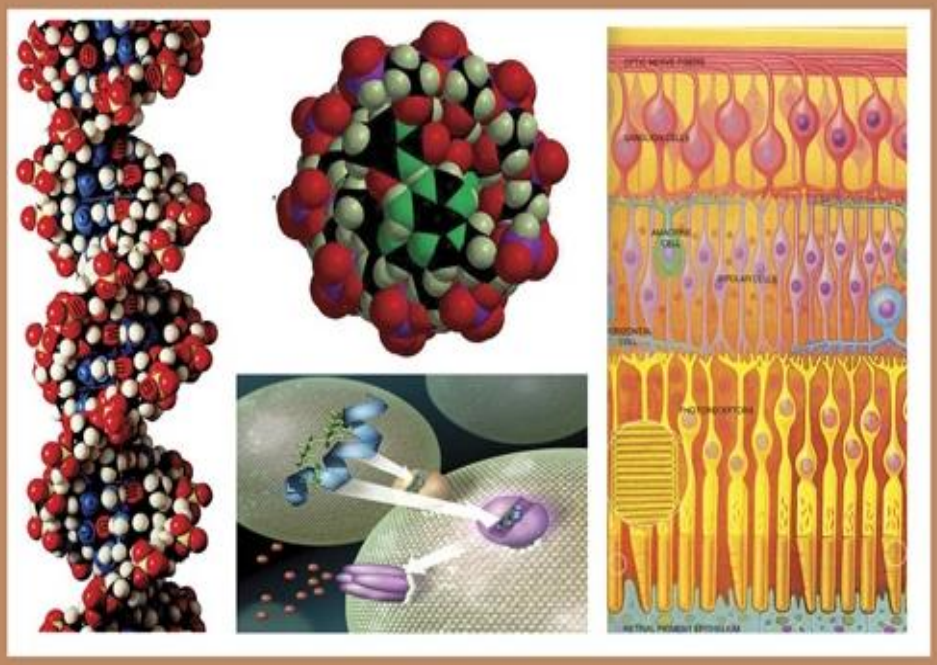

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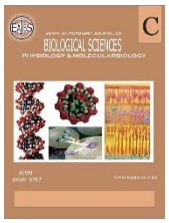

Effect of 5-Azacytidine as A Demethylation Agent on PCNA and RB1 Genes in Hepatocellular Carcinoma Cell Model "HepG2"

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ABSTRACT

Epigenetic alterations are associated with human cancer development and the inactivation of tumor suppressor genes. Two such tumor suppressor genes, PCNA and RB1, showed an aberrant gene expression in HCC. DNA methylation alters the expression of genes and is one of the processes that transform normal cells into cancer cells. PCNA and RB1 genes screened methylation in the promoter regions in HepG2 cells. The current study examined the effect of DNA methyltransferase inhibitor (5-Azacytidine) on gene expression of PCNA and RB1 genes after treatment and showed the impact of 5-aza-CR on the Methylation degree of HepG2 cells. HepG2 cell line originated from hepatocellular carcinoma (HCC). Since HepG2 exhibits the characteristics of human liver carcinoma, it was a good model for detecting the changes in methylation patterns and the gene expression level that was detectable in a clinical setting. The human HepG2 cell line was treated with 5, 10, and 25 µM of 5-aza-CR for 24 h, 48 h, and 72 h. Methylation of PCNA and RB1 was detected by methylation-specific polymerase chain reaction (MSP). PCNA and RB1 gene expression detected by reverse transcription-polymerase chain reaction. The influence of 5-aza-CR on Cell viability was assessed by SRB assay for 24 h, 48 h, and 72 h. The IC50 is 20.52 µM for 24 h, 12.6117 µM for 48 h, and 10.63 µM for 72 h after exposure to 5-aza-CR, which showed that 5-aza-CR inhibited the growth of HepG2 cells in a time, and dose-dependent manner. Although other genes may be demethylated due to the 5-aza-CR treatment, we concentrated on the PCNA and RB1 genes. In HepG2 cells, PCNA and RB1 gene methylation were found before 5-aza-CR treatment. In contrast, no PCNA or RB1 gene expression was detected. Treatment with different concentrations of 5-aza-CR significantly decreased the methylation degree of the PCNA and RB1. 5-aza-CR at 25 µM for 72h showed the highest induction activity of PCNA and RB1 gene expression. Methylation-specific PCR results showed that 5-aza-CR promoted the expression of PCNA and RB1 by demethylation. Our results illustrate that 5-aza-CR could reverse the abnormal methylation degree of the PCNA and RB1 genes that are hypermethylated in HepG2 cells and induces the expression of the PCNA and RB1 genes by demethylation.

INTRODUCTION

Hepatocellular carcinoma(HCC) is the most lethal type of liver cancer disease (Sung *et al*., 2021.; Huang *et al*., 2021). It is the third leading cause of cancer-related death in the world. The last statistical evaluation by the WHO update reported that HCC has recorded 905.677 new incidences diagnosed and 830.180 deaths worldwide for both males and females (Sung *et al*., 2021). HCC has primarily associated with HBV and HCV virus infection, excessive alcohol consumption, liver cirrhosis, and epigenetic alteration such as DNA methylation (Wolinska and Skrzypczak, 2021).

Epigenetics is the study of heritable gene expression levels without affecting the DNA sequence. HCC is related to epigenetic alterations like DNA hypermethylation, DNA hypomethylation, dysregulation of histone modification levels, and chromatin remodeling (Wang *et al*., 2021). Many studies have demonstrated that liver cancer initiation, clinical diagnosis, prognosis, and treatment are associated with DNA methylation (Laugsand *et al*., 2015; Liu *et al*., 2020). DNA methylation includes adding a methyl group to cytosine in CpG dinucleotides in the promoter and regulatory regions (Toh *et al*., 2019). DNA methylation is regulated by DNMT enzymes composed of DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Bestor, 2000; Okano *et al*., 1998).

Aberrant DNA methylation of tumor suppressor genes promoters has been observed in HCC (Bhat *et al*., 2018; Dong and Wang, 2014). Oncogene hypomethylation and tumor suppressor gene hypermethylation are two examples of methylation changes that are thought to be essential events in cancer development, including HCC *(*Huang *et al*., 2011; Lambert *et al*., 2011; Long *et al*., 2019). Genes correlated to cell cycle regulation, apoptosis, DNA repair, carcinogen metabolism, and angiogenesis are frequently influenced by aberrant hypermethylation in the promoter regions (Esteller, 2007).

The first tumor suppressor (TSG) gene identified is the retinoblastoma (RB1) gene. It is frequently inactivated in different types of cancers (Chen *et al*., 2009; Tang *et al*., 2017). However, the Rb protein is typically downregulated in HCC cells. Numerous studies on the downregulation of RB1 expression in HCC have been suggested, including genetic loss, epigenetic abnormalities, and post-transcriptional degradation (Anwar *et al*., 2014; Laurent-Puig and Zucman-Rossi, 2006). Recent studies revealed that Aberrant DNA methylation at the RB1 gene leads to dysregulation of gene expression of an alternative RB1-E2B transcript (Anwar *et al*., 2014). RB1 controls the E2 factor (E2F) family of transcription factors, acting as a negative regulator of cell cycle progress (Qin *et al*., 2004).

The proliferation of cell nuclear Antigen(PCNA) is essential in the development and progression of cancer (Cheng *et al*., 2020). It is a 36,000-kDa protein that acts as a DNA sliding clamp and regulates cell proliferation (Biasio and Blanco, 2013). According to previous studies, PCNA regulates proliferation by acting as a scaffold to recruit proteins involved in DNA replication, repair, cell-cycle control, survival, and chromatin assembly (Moldovan *et al*., 2007). It has been demonstrated that proliferating cell nuclear antigen is tightly associated with the proliferation of cancer cells, including HCC cells (Chen *et al*., 2018).

5-Azacytidine is clinically approved by the FDA and the European Medicines Agency that act as a demethylating agent to treat acute myeloid leukemia (AML), chronic myelomonocytic leukemia (CMML), and severe myelodysplastic syndrome (MDS) (Venturelli *et al*., 2013). The demethylating agent 5-Azacytidine inhibits DNMT enzymes and reverses DNA methylation (Patra and Bettuzzi, 2009). The methylation pattern of PCNA and RB1 in HepG2 cells with 5-aza-CR has not been studied. In this study, we probed to

investigate the effect of 5-aza-CR on PCNA and RB1 gene expression and methylation level of HepG2 cells.

MATERIALS AND METHODS

1. Cell line Culture:

HepG2 cell line (ATCC, Manassas, VA, USA) was seeded as monolayers in 75 cm^2 flasks cell culture (Falcon, Corning, NY, USA) contained Dulbecco minimum Eagle's medium (DMEM; Sigma, Irvine, UK) medium consists of 10 % of heated fetal bovine serum (FCS; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and 100 mg/mL of streptomycin, 100 units/mL of penicillin incubated in a humidified incubator containing 5% Co2 at 37°C. 5-aza-CR (Celgene) was dissolved in complete media and diluted to target concentrations of 5, 10, and 25 μM in the culture fluid. Cells were treated with different concentrations of 5-aza-CR for 24, 48, or 72 h.

2. Cytotoxicity Assay and Cell Viability:

The viability of HepG2 cells was assessed via SRB assay to calculate IC50 for 5-Azacytidine in HepG2. Approximately ($5x10^3$) were seeded in 96- microtiter plates containing a complete media for 24h. Another 100μL medium containing drug at various concentrations (0-100 M) was used to treat the cells. 150μL of 10% TCA was added to cells after 24, 48, and 72 h of drug exposure for fixation and maintained at 4°C for 1 h. Distilled water is used for washing five times after removing the TCA solution. In a dark place at room temperature for 10 minutes, 70μl SRB solution (0.4 % w/v) was added and maintained. After incubation, the plate was washed three times with 1% acetic acid. Then, the plate was removed and dried by air overnight. The proteins that were bound to SRB were dissolved by adding 150μL of TRIS (10mM). The Microplate reader Molecular Device (Ortenberg, Germany) (Allam *et al*., 2018; Skehan *et al*., 1990) was used to measure the optical density. The half maximal inhibitory concentration (IC50) values were also calculated using statistical Graph pad prism software.

3. 5-Azacitidine Treatment:

Approximately (1×10^5 /mL) cells in experimental groups were seeded in 6 well plates and the treatment involved 0, 5, 10, and 25 μM of 5-aza-CR (Celgene) based on SRB result in cultured fluid in different incubation times: 24, 48, and 72 h. The treated medium was changed after 24,48 and 72 h. The cells were cultured for seven days to recover from the toxicity of 5-aza-CR. Untreated cells were analyzed under similar conditions as a control.

4. RNA Extraction And Formation of cDNA:

RNA was isolated via TirRNA Pure Kit (Geneaid Biotech Co). The RT-PCR equipment was used to reverse extracted RNA per the manufacturer's instructions. Reverse transcriptase reactions happened with a one-step SYBR Green mix (Enzynomics, Daejeon, Korea), and Gene-specific primers were designed from the primer Bank. The primers sequence used for PCNA, RB1 gene, and β -Actin (reference gene) are listed in Table 1. cDNA was synthesized using qRT-PCR and amplified. The PCNA and RB1 expression level were analyzed using the LightCycler 480 (Roche Diagnostics, Nutley, NJ, USA). The measurements were calculated in triplicate. The gene expression ratio was calculated by the following equation 2^-ΔΔCt, in which ΔΔCt = (Ct target gene-Ct β -Actin gene) target sample - (Ct target gene -Ct β-actin gene) Calibration sample.

Table 1. primer sequence listed for RT-PCR

Gene Name	Forward	Reverse
PCNA	CAGTTCCCTTAGCAGCCCAG	AATCGCACACTGAAACGCAC
RB1	AGGTGGTGATGGTGATGCTAC	TTCTAGCTGAGCAGGGAACA
β -Actin(reference)	GCACCACACCTTCTACAATG	TGCTTGCTGATCCACATCTG

5. DNA extraction and Detection of methylation pattern:

Genomic DNA was isolated from the control and HepG2 treated cells using a cultured cell extraction kit (Geneaid Biotech Co.) according to the manufacturer's instructions. Extracted gDNA was measured by NanoDrop \ (Molecular device) and modified by Bisulfite kit (Zymo Research, Irvine, USA). The methylated primer sequence used for methylation analysis of PCNA and RB1 is listed in Table 2

.

Table 2. primer sequence listed for MSP-PCR

Gene Name	Forward	Reverse	Product size	Annealing Tm °C
PCNA	M: TGGTTAATCGTATATTGAAACGTAC	GAAAACAAAACTCGAAACGAA	191	56.5
	U: TGGTTAATTGTATATTGAAATGTATGT	CCAAAAACAAAACTCAAAACAAA	193	55.3
RB1	M: TTTAAAATTTTTCGAAAAACGGTC	AAACGACGACGACTCTACTCG	157	59.7
	U: TTTAAAATTTTTTGAAAAATGGTTGT	TAAAACAACAACAACTCTACTCACT	159	54.3

6. Statistical Analysis:

Statistical analysis was performed using a Graph pad prism, The experiment was repeated at least three to five times. Two ways of ANOVA analysis of variance, T-test, and Chi-Square, were used to examine differences between experimental groups. The data are presented as mean ± standard deviation (SD), with $P < 0.05$ considered statistically significant.

RESULTS

1.Cytotoxic Activity Of 5-aza-CR:

Cytotoxic activity of 5-aza-CR on human hepatocellular carcinoma HepG2 cell line compared with the control, 5-aza-CR inhibited HepG2 cell growth, and the inhibition was dose and time-dependent. The IC50 was calculated to be 20.52 µM at 24 h, 12.6117 µM at 48 h, and 10.63 µM at 72 h, so we selected the 5, 10, and 25 µM concentrations to rule out the cytotoxicity (Fig. 1).

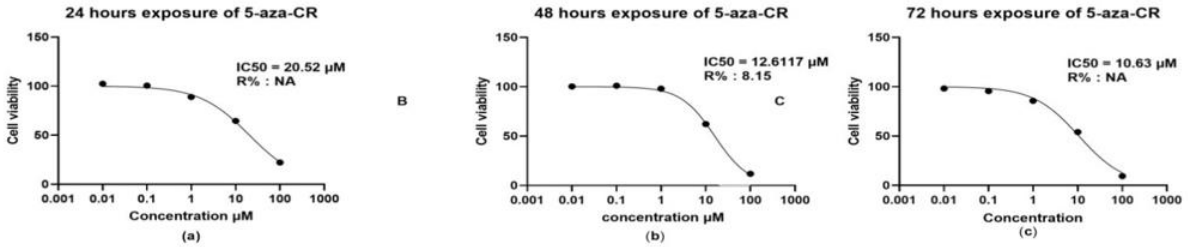

Fig. 1: This scheme shows cell viability results in which (a) shows SRB result after 24 h exposure of 5-aza-CR and, IC50 is 20.52 µM (b) shows SRB result after 48 h exposure of 5-aza-CR and IC50 is 12.6117 µM and, (c) shows SRB result after 72 h exposure of 5-aza-CR and IC50 is 10.63 µM.

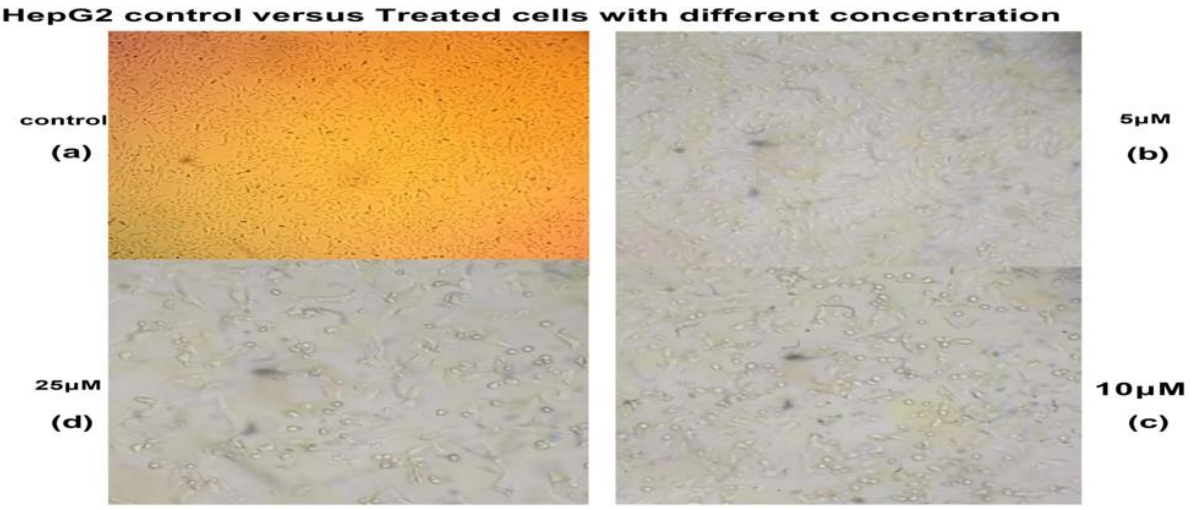

Fig. 2: HepG2 cells control (a) versus treated cells with different concentrations of 5-aza-CR (b) shows cell viability with 5 µM, (c) shows cell viability with 10 µM, and (d) shows cell viability with 25 µM.

2. Methylation Pattern Analysis:

To investigate the methylation degree in RB1 and PCNA in the promoter region and how it affects gene expression levels, we designed an unmethylated primer confirming the unmethylation degree and a methylated primer confirming the methylation degree. Primers were designed according to the Methprimer manual. The primer sequence and PCR condition are listed in Table 2; lane U refers to unmethylated primer and lane M refers to Methylated primer. Because unmethylated primers of RB1 and PCNA genes were used in the control group of HepG2 cells, DNA was not amplified. However, after treatment, the DNA amplification increased depending on the 5-aza-CR concentration and long different time exposure. RB1 and PCNA DNA were amplified when Methylated primers were used in the control group of HepG2 cells. These results illustrate that 5-aza-CR demethylates the methyl groups gradually in treated cells of HepG2 cells.

PCNA and RB1 gene methylation was detected in the control group of HepG2 cells. In the PCNA gene, the methylation pattern was detected in 6 replicates for each concentration. The results showed that after 24 hrs of treatment, the methylation percent decreased with the increase of the concentration of 5-aza-CR at (25 µM) to (0.0%) when compared with the control group (p. value 0.001). This percentage was confirmed by results after 48 and 72 hours of treatment, while those were (0.0 %) at a concentration of 25 uM for both times (Table 3).

Interestingly, the partially methylated percent also changed to (0.0%) after 72 hrs of the treatment, which is (25 uM) less than 24 and 48 hrs. Therefore, we can conclude that 5-aza-CR can have an effect on the methylation pattern after treatment for 72 hrs (Table 3).

Regarding the RB1, the results showed that the methylation percent decreased but was not hidden after 24hrs of treatment (25 uM), while after 48 hr, the methylation pattern decreased and completely hidden (0.0%) with (10 and 25uM) when compared with the control group (p. value 0.001) (Table 4).

Table 3: The table shows the Methylation pattern of the PCNA gene with 5-aza-CR treatment in 24,48 and 72 h.

Time points	5-Aza C Conc.	Un-methylated N (%)	Partially N (%)	Methylated N (%)	P. value
	control	0%	0%	100%	
24 hrs	5 µM	33.33%	66.67%	0%	<0.0001
	10 µM	33.33%	33.33%	33.33%	<0.0001
	25 µM	0%	100%	0%	<0.0001
48 hrs	5 µM	0%	100%	0%	<0.0001
	control	0%	0%	100%	
	10 µM	0%	100%	0%	<0.0001
	25 µM	33.33%	33.33%	33.33%	<0.0001
	control	0%	0%	100%	
72 hrs	5 µM	0%	66.70%	33.33%	<0.0001
	10 µM	100%	0%	0%	<0.0001
	25 µM	33.33%	33.33%	33.33%	<0.0001

The results of the studied time points are represented as percent (%); the data were analyzed by X^2 test.*P value < 0.05 is significant, *P value < 0.01 is highly significant as compared to control group.

Table 4: The table shows the Methylation pattern of the RB1 gene with increasing 5-aza-CR in 24,48 and 72 h.

Time points	5-Aza C Conc.	Un-methylated N(%)	Partially N(%)	Methylated N(%)	P. value
Control	0 µM	0%	33.33%	66.67%	<0.0001
24 h	5 µM	0.00%	100.00%	0%	<0.0001
	10 µM	0.00%	100%	0.00%	<0.0001
	25 µM	0%	67%	33.33%	<0.0001
	control	0%	0%	100%	
48 h	5 µM	0%	33%	66..67%	<0.0001
	10 µM	33.33%	67%	0	<0.0001
	25 µM	66..67%	33.33%	0	<0.0001
	control	0%	0%	100%	
72 h	5 µM	33.33%	66.67%	0.00%	<0.0001
	10 µM	0%	100%	0%	<0.0001
	25 µM	0.00%	66.67%	33.33%	<0.0001

The results of the studied time points are represented as percent (%); the data were analyzed by X^2 test.*P value < 0.05 is significant, **P value < 0.01 is highly significant as compared to control group.

3. Expression of Studied Genes:

The gene expression level was detected and evaluated according to the Fold-Chang law, which depends on the threshold of the housekeeping gene and the control group. Therefore, the control group's results should equal one to determine the gene expression direction after treatment. The gene expression of PCNA after treatment with (25 µM) 5-aza-CR showed that it increased to (1.5±0.15) after 24 hrs of treatment, (1.6±0.33) after 48 hrs, and (2.8±2.7) after 72 hrs (Fig. 1A). Also, the gene expression of RB1 after treatment with (25 µM) 5-aza-CR showed that it increased to (2±0.5) after 24 hrs of treatment, (3.6±1) after 48 hrs, and (4.2±1.3) after 72 hrs (Fig. 1B and Table 5).

Hence, the gene expression of the studied genes (PCNA and RB1) showed a gradual increase in gene expression with time after treatment with (25 µM) 5-aza-CR, indicating that the gene expression would be accessible after the deletion of the

methylation effect by the 5-aza-CR agent in the hepatocellular carcinoma cell model.

The correlation study confirms the relations between the demethylation effect of 5-azaC at (25 µM) at different times with the gene expression of the studied genes during the same different times, which was (r=0.678, p.value 0.001**) between the reduction of methylation percent and the increase in the gene expression of PCNA, while (r=0.686, p.value 0.001**) in the case of RB1.

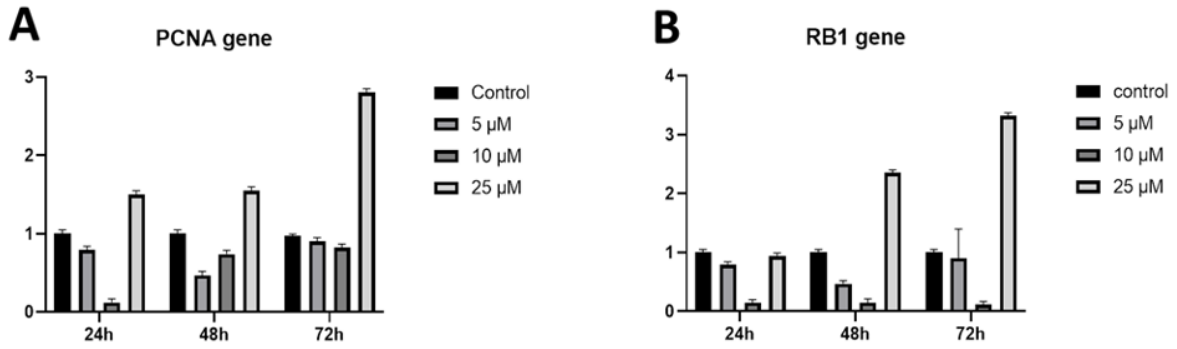

Fig. 3: Gene expression level of studied genes. Figure (A) is PCNA gene expression, and Figure (B) is RB1 gene expression.

Table 5. Gene Expression of studied genes**.**

Time points	5-Aza C Conc.	PCNA		RB1	
		Mean±S.D	P value	Mean±S.D	P value
Control	0 µM	1±0.01	-	1±0.01	-
24 h	5 µM	0.8±0.28	<0.0001	0.8±0.3	<0.0001
	10 µM	0.1±0.39	<0.0001	0.2±0.01	<0.0001
	25 µM	1.5±0.15	<0.0001	2±0.5	<0.0001
48 h	5 µM	0.5±0.38	<0.0001	0.5±0.4	<0.0001
	10 µM	0.7±0.19	<0.0001	0.2±0.01	<0.0001
	25 µM	1.6±0.33	<0.0001	3.6±1	<0.0001
72 h	5 µM	0.9±4.2	<0.0001	0.9±0.1	<0.0001
	10 µM	0.8±1.36	<0.0001	0.1±0.1	<0.0001
	25 µM	2.8±2.7	<0.0001	4.2±1.3	<0.0001

DISCUSSION

RB1 and PCNA showed gene abnormalities in developing HCC (Laurent-Puig, Zucman-Rossi, 2006 and Anwar *et al*., 2014). In the present study, we investigated the effect of 5-aza-CR on the gene expression level of PCNA and RB1 in the HepG2 cell line. Various factors interrupt RB1 and PCNA gene expression in HCC; stimulating factors are necessary. In this study, cell viability was assessed by SRB assay, and IC50 was calculated of 5-aza-CR in 24, 48 and 72 h in HepG2 cells. Control cells showed that no gene expression was detected in RB1 and PCNA. MSP-PCR was performed and indicated the presence of methylation patterns in control cells. RB1 and PCNA are suppressed at 5,10µM of 5-aza-CR treatment with no significant change as a control, while the expression induced at 25 µM and increased in long different incubation times in PCNA and RB1.

Epigenetics is the study of heritable gene expression without affecting the DNA sequence. HCC is related to epigenetic alterations like DNA hypermethylation or hypomethylation, dysregulation of histone modification patterns, and chromatin remodeling (Wang *et al*., 2021). DNA methylation involves the

addition of methyl group to cytosine in CpGdinucleotides in the promoter region and regulatory region (Nagaraju *et al*., 2021). DNA methylation is regulated by DNMT enzymes composed of DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Bestor, 2000) (Okano *et al*., 1998). De novo DNMTs enzymes change the methylation degree of several genes like p53 (Tate and Bird, 1993) (Wang *et al*., 2005) (Varela *et al*., 2007). In Hep3B, P53 is not expressed. However, 5-aza-CR, a demethylating agent, induces the expression of the P53 gene and induces apoptosis (Varela et al., 2007). Interestingly, 5-aza-CR activated p15 and p 16, which can stop the proliferation (Dong and Wang, 2014). UCP2 is not expressed in Hep3B and HT-29 as a result of the degree of methylation in the promoter region. However, 5-aza-CR increased the expression level as a result of the effect of a demethylating agent (Kim *et al*., 2021).

In our study, when HepG2 cells that do not express RB1 and PCNA were treated with 5-aza-CR, a demethylating agent (Hurtaud *et al*., 2007). PCNA and RB1 are induced by DNA demethylation. According to the previous studies and our result, we thought that 5-aza-CR induces the expression of RB1 and PCNA genes. Figure 3 and Table 4 showed that 5-aza-CR inducing gene expression at 25 μM of PCNA and RB1 in HepG2 as compared to control.

We hypothesize that RB1 and PCNA gene expression is induced in HepG2 cells based on the results presented in tables 3, 4, and 5 by DNA demethylation. The level of expression may change depending on the methylation degree. On the other hand, the degree of methylation change correlated with inducing gene expression. Treated cells at 5,10 μM showed no significant difference as compared to control may be due to the following reasons; RB1 and PCNA may be inconsistent in hepatocellular carcinoma; the expression levels of PCNA and RB1 are correlated with environmental factors (e.g., the concentration of 5-aza-CR) and the utilized method for detection may affect the experimental results. This conflict reflects the complexity of RB1 and PCNA gene expression levels in hepatocellular carcinoma.

The control group and 5,10μM treated HepG2 cells showed that RB1 and PCNA gDNA was not amplified due to the degree of methylation present in the promoter region; however, treatment with 5-aza-CR at 25 μM significantly induced RB1 and PCNA gDNA. Further studies are necessary to detect the methylation level for the RB1 and PCNA promoter regions.

In HCC cells, RB1 and PCNA play a vital role in proliferation. RB1 controls the E2 factor (E2F) family of transcription factors, acting as a negative regulator of cell cycle progression. In contrast, PCNA regulates proliferation by acting as a scaffold to recruit proteins involved in DNA replication, repair, cell-cycle control, and survival, as well as chromatin assembly(Cheng, *et al*., 2020). In the Future, Further studies are needed to investigate the methylation level for the entire UCP2 promoter region.

5. Conclusions

The PCNA and RB1 promoter in HepG2 cells are methylated in the case of hepatocellular carcinoma and the degree of methylation in the promotor regions of PCNA and RB1changed with increasing 5-aza-CR concentrations. Therefore; the 5-aza-CR agent can increase the expression of the tumor suppressor genes (PCNA and RB1) in the HepG2 cell model. Then we can conclude that the 5-aza-CR can be used as a demethylation agent also in hepatocellular carcinoma.

6. Abbreviations:

5-aza-CR: 5-Azacytidine; cDNA: complementary DNA; DMEM: Dulbecco's mini-mum Eagle's medium; DNMTs: DNA methyltransferases; FCS: fetal calf serum; TSG: Tumor suppressor gene; gDNA: genomic DNA; HCC: hepatocellular carcinoma; MSP: methylation-specific PCR; PCNA: proliferating cell nuclear antigen; RB1: retinoblastoma 1.

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Institutional Review Board Statement:

The study was performed in accordance with the Declaration of Saudi Arabia and approved by the ethics committee of the department of biological science of King Abdelaziz university.

Informed Consent Statement: Informed consent to participate in the study was obtained from all authors who contributed to this study.

Data Availability Statement: The data is available on reasonable request to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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