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Decitabine attenuates the anti-cancer effect of doxorubicin by repressing ECDH1 and inducing SNAI1 and BCL2 in HepG2 hepatocellular carcinoma cells.

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Abstract

Purpose: Methylation is an epigenetic mechanism acting as a barrier for successful chemotherapy. Decitabine is a demethylating agent recently used in treating cancer. However, its effect on hepatocellular carcinoma (HCC) has not been well understood. This work aims at evaluating the action of decitabine single and in combination with doxorubicin on methylation and gene expression in HepG2 cell line.

Methods: Methylation of SNAI1 and ECDH1 and expression of ECDH1, NCDH1, SNAI1, BCL2, BAX and TWIST were studied on HepG2 cells treated with decitabine, doxorubicin and dec./dox. combination. Treated cells were tested for apoptosis and cell cycle analysis by flow cytometry.

Results: Decitabine induced ECDH1 methylation and SNAI1 demethylation. Higher ECDH1 methylation was significantly correlated to lower SNAI1 methylation (P = 0.0001). The result was confirmed by high SNAI and low ECDH1 expressions. Decitabine and combination treated cells showed high BCL2 and BAX2 expressions. TWIST and NCDH1 were downregulated in all treated cells. ECDH1 expression was significantly correlated to NCDH1 expression (P= 0.0001). SNAI1 expression was significantly correlated with BCL2 expression (P< 0.00001). Inhibition of apoptosis was noticed in decitabine treated cells. doxorubicin and combination treated cells showed necrotic cell death. Doxorubicin was significantly better than decitabine (P= 0.0061) and sequential combination (P=0.00043) in inducing G2 arrest.

Conclusion: Decitabine induce EMT-related transcription factor SNAI1 and the antiapoptotic factor BCL2 in HepG2 cells. Decitabine attenuate the action of doxorubicin through activating EMT and inhibition of apoptosis. Decitabine may attenuate the action of doxorubicin if both drugs combination was applied for HCC treatment.

Keywords: Decitabine, Doxorubicin, Hepatocellular carcinoma; HepG2, Methylation, EMT, CDH1, SNAI1.

1. Introduction

Liver cancer is the 5th most common cancer worldwide and the third most common cause of cancer related death. HCC is one of the most aggressive tumors accounting for about 85% of liver primary tumors, with approximately 598,000 deaths each year, worldwide (1). In Egypt, HCC is the second most frequent cause of cancer incidence and mortality in men (1). Hepatitis C virus (HCV) infection is considered as an etiological factor for HCC. Egypt constitutes the highest infection rate of HCV accordingly; Egypt exhibits an increasing rate of HCC (2). Most of HCC patients are diagnosed at a late stage of the disease which makes the treatment is difficult. This is in addition to the resistance of the disease to existing therapies (3). Surgical therapy remains the best curative modality but because most of the patients are presented with an advanced stage of the disease the chance of tumor resection is low (4). Due to the resistance of HCC to the available chemotherapeutic agents, there is an urgent need to discover new treatments and develop new methods of therapy to improve the low survival rates of this disease. These methods include targeting cancer

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pathways or therapies that target the epigenetic processes in liver cancer.

Epigenetics is the change of gene expression without altering the coding sequence of the gene (5). Epigenetic processes include DNA methylation, histone modification and noncoding RNA-mediated processes (5). Recent studies indicated that defective epigenetic processes are considered a hallmark of cancer (6). Defective epigenetic mechanisms have been linked to tumor aggressiveness (7), metastasis (8), recurrence and resistance to chemotherapy (9). Recent studies indicated that hypermethylation is responsible for the silencing of tumor suppressor genes (10) which may activate epithelial mesenchymal transition (EMT) (11) and antiapoptotic function (12 & 13) which may lead to malignant transformation. Accordingly, hypermethylation of cancer cells is an attractive target for cancer therapy.

One of the well-known hypomethylating agents is Decitabine (4-amino-1-(2-deoxy-D-ribofuranosyl)-1,3,5-triazine-2(1H)-one). It directly attaches to DNA strands and inhibits DNA methyltransferase activity. Decitabine was authorized for the treatment of acute myeloid leukemia (AML) in addition to being used to treat myelodysplastic syndromes (MDS). Decitabine may be used in combination with other anti-tumor agents to increase their effectiveness against some types of cancers, including HCC (14). Increasing the rate of apoptosis was observed in HCC cell lines when decitabine was used in combination with trichostatin-A via restoring the expression of delta-like 3 (15). Furthermore, after HepG2 cells were treated with decitabine, overexpression of the general receptor for phosphatides associated scaffold protein (GRASP) was observed as a result of its methylation being downregulated (16)..

The aim of the present study is to test the efficacy of decitabine alone or in combination with doxorubicin on HepG2 HCC cell line and to detect the impact of hypomethylation resulting from decitabine on the expression of genes related to adhesion, including E-CDH1 and N-CDH1, epithelial to mesenchymal transition, including SNAI1, and apoptosis, including Bcl2 and Bclx.

2. Materials and Methods:

Cell line:

The human HCC cell line (HepG2), obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt), was cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% of heat-inactivated fetal bovine serum (Gibco, Life technologies Inc., UK) at 37ºC in a humidified 5% CO2 incubator (Thermo Scientific, City, USA). Decitabine was obtained from MYLODEC (CELON LABS, Belgium), Doxorubicin was obtained from Sigma Aldrich (Merck KGaA, Germany).

Cell viability assay:

Cell viability was assessed by Sulpho Rhodamine-B assay (SRB) as described previously (Skehan et al., 1990) (17). In brief, aliquots of 100µL cell suspension (5x103cells) were seeded into 96-well plates and incubated in complete media for 24h. Cells were treated with another aliquot of 100µL media containing drugs (Decitabine / Doxorubicin) at various concentrations ranging from (0.01, 0.1, 1, 10, 100µM). After 72h of drug exposure, cells were fixed by replacing media with 150µL of 10% TCA and incubated at 4°C for 1h. The TCA solution was removed, and the cells were washed 5 times with distilled water. Aliquots of 70µL SRB solution (0.4%w/v) were added and incubated in a dark place at room temperature for 10 min. Plates were washed 3 times with 1% acetic acid and allowed to air-dry overnight. Then, 150µL of TRIS (10mM) was added to dissolve the protein-bound SRB stain. The absorbance was measured at 540nm using a BMG LABTECH-FLUO star Omega micro-plate reader (Ortenberg, Germany). Each treatment was done in triplicates. The IC50 for each drug was calculated. Accordingly, concentrations of 5, and 10 µM for Decitabine while 0.6 µM for Doxorubicin were used for subsequent studies.

Promoter methylation analysis:

Extraction of DNA from cells was done using QIAamp Fast DNA Tissue Kit (Qiagen, Germany). For each sample the following cocktail was added: 200 µl AVE, 40 µl VXL, 1 µl DX Reagent, 20 µl proteinase K, and 4 µl RNase A (100 mg/ml). Samples were homogenized by vortex for 5 min, and incubated in a thermomixer at 1000 rpm for 10 min at 56ºC. Then 265 µl Buffer MVL was added and mixed by vortexing. The mixture was transferred to the QIAamp Mini spin column and processed according to the manufacturer's instructions. DNA concentration and purity were measured using a Nanodrop One spectrophotometer (Thermo- scientific Fisher, USA).

Promoter methylation for E-Cadherin 1 (ECDH1) (EPHS105415-1A) and SNAI1 (EPHS109344-1A) was studied using the Methyl Screen technology by EpiTect Methyl II Primer Assay kits (Cat. no. 335002) (Qiagen, Germany). The restriction digestions were performed using the EpiTect Methyl II DNA Restriction Kit (Cat. no. 335452) (Qiagen, Germany). Amplification was performed using an Applied Biosystem ViiA7 real-time PCR instrument (Thermo- scientific Fisher, USA). The PCR program was performed according to the manufacturer's instructions. Analysis was performed using the

dedicated EpiTect Methyl II PCR Array Microsoft. Excel template (www.sabiosciences.com/dna_methylation_data_analysis.php). Briefly, the CT values were exported to the data analysis sheet and the results (percentage of promoter methylation for each gene) were automatically generated.

RNA isolation, reverse transcription (RT) and quantitative PCR (qPCR) assay:

Total RNA was isolated from cells using miRNeasy Micro Kit (Cat. no. 217084) (Qiagen, Germany) according to the manufacturer's recommendations. RNA concentrations and purity were determined using a NanoDrop One spectrophotometer (Thermo- scientific Fisher, USA). The RT2 First Strand Kit (Cat. no. 330404) (Qiagen, Germany) was used for cDNA synthesis from isolated total RNA following the manufacturer's instruction. Briefly, 5µl of genomic DNA elimination buffer (GE) was added to the RNA and completed to 10µl. The reagents were mixed well and incubated at 42°C for 5 minutes then chilled on ice for at least 1 minute. The RT cocktail was prepared by adding 4µl RT buffer (BC3), 1µl primer and external control mix (P2), 2µl RT enzyme mix (RE3) then completed to 10µl by adding 3µl of RNase-free water. 10µl of RT cocktail were added to10µl genomic DNA elimination mixture. The cocktail was mixed well and incubated at 42°C for 15 minutes. The reaction was stopped by heating at 95°C for 5 minutes. 91µl of RNase-free water was added to the reaction. Gene expression analysis was performed using SYBR Green RT-PCR Kit (Cat. no. 330504) (Qiagen, Germany). The primers of ECDH1, SNAI1, NCDH1, BCL2, BAX and TWIST were obtained from (Eurofins Genomics, Germany). A total of 10 µl reaction volume was done as follows: 5 µl SYBR green PCR master mix, 1 µl of 10 µM Forward primer, 1 µl of 10 µM Reverse primer, 1 µl template cDNA, 2 µl RNase-free water. Each amplification reaction was performed in duplicate on a ViiA 7 real-time PCR instrument (Thermo- scientific Fisher, USA). qPCR protocol consisted of a denaturation step at 95°C for 10 min, followed by 40 amplification cycles at 95°C for 15 s and 60°C for 1 min. GAPDH was used as the internal control. Relative expression for each sample was calculated using the $2^{-\Delta\Delta CT}$ method.

Apoptosis analysis by flow cytometry:

Apoptosis and necrosis cell populations are determined using Annexin V-FITC apoptosis detection kit (Abcam Inc., Cambridge Science Park, Cambridge, UK) coupled with 2 fluorescent channels flow cytometry. After treatment with decitabine (5µM and 10 µM), doxorubicin (0.6µM) and the combination of both drugs for 24/48 and 72 h, cells (10^5 cells) are collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Then, cells are incubated in the dark with 0.5 ml of Annexin V-FITC/PI solution for 30 min in dark at room temperature according to manufacturer protocol. After staining, the cells are injected via ACEA Novocyte™ flowcytometer (ACEA Biosciences Inc., San Diego, CA, USA) and analyzed for FITC and PI fluorescent signals using FL1 and FL2 signal detector, respectively (λex/em 488/530 nm for FITC and λex/em 535/617 nm for PI). For each sample, 12,000 events are acquired and positive FITC and/or PI cells are quantified by quadrant analysis and calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA).

Cell cycle analysis by flow cytometry:

After treatment with decitabine (5µM and 10 µM), doxorubicin (0.6 µM) and the combination of both drugs for 72 h, cells (105 cells) are collected by trypsinization and washed twice with ice-cold PBS (pH 7.4). Cells are re-suspended in two milliliters of 60% ice-cold ethanol and incubated at 4ºC for 1 h for fixation. Fixed cells are washed twice again with PBS (pH 7.4) and re-suspended in 1 mL of PBS containing 50 µg/mL RNAase A and 10 µg/mL propidium iodide (PI). After 20 min of incubation in the dark at 37 C, cells are analyzed for DNA contents using flow cytometry analysis using FL2 (λex/em 535/617 nm) signal detector (ACEA Novocyte™ flow cytometer, ACEA Biosciences Inc., San Diego, CA, USA). For each sample, 12,000 events are acquired. Cell cycle distribution is calculated using ACEA NovoExpress™ software (ACEA Biosciences Inc., San Diego, CA, USA).

Statistical analysis:

Statistical analysis was done using IBM SPSS Statistics version 22 (IBM Corp., Armonk, NY, USA). Numerical data were expressed as median and range. Qualitative data were expressed as frequency and percentage. Pearson's Chi-square test or Fisher's exact test was used to examine the relation between qualitative variables. Quantitative data were tested for normality using the Kolmogorov-Smirnov test and Shapiro-Wilk test. The data were found to be not normally distributed. Comparison the two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between the three groups was done using Kruskal-Wallis test (non-parametric ANOVA). Spearman's Rho method was used to test the correlation between numerical variables. All tests were two-tailed. A p-value < 0.05 was considered significant.

Results:

Effect of DOX and DAC on the HepG2 cell viability:

The growth inhibitory effect of DOX and DAC on the HepG2 cell line was assessed using SRB assay as illustrated in Figure 1A. DOX showed a significant growth inhibitory effect on HepG2 in a

concentration-dependent manner as compared to control untreated cells. Its IC50 value was 0.6 uM. However, the treated cells by different doses of DAC showed a poor cytotoxic effect at low doses as presented in Figure 1B. Based on the present data, low doses of DAC (5, and 10 uM) can be used as a demethylating agent without any cytotoxic effect on HepG2 cells in the subsequent experiments.

Effect of DAC (5 and 10 uM) on methylation status of CDH1 and SNAI1:

The present data demonstrated that the promoter for SNAI1 of untreated control HepG2 cells was absolutely methylated and the degree of methylation decreased in the presence of DAC at 5 uM concentration (< 0.00001). The percentage of methylation elevated close to the untreated cells in the presence of 10 uM concentration of DAC as shown in Figure 2A and table1 (0.007369). Then the degree of methylation was decreased in the presence of DAC/DOX combination (< 0.00001). However, the methylation status of CDH1 promoter demonstrated unexpected results where high methylation (>80%) was reported in the presence of DAC 5 (< 0.00001) and 10 uM (< 0.00001) comparing with the absence of DAC as illustrated in Figure 2B and Table 2. The methylation of SNAI1 was correlated to that of ECDH1 in DAC treated cells. At a concentration of 5 μM, high methylation level of ECDH1 was significantly associated with low methylation level of SNAI1 (p<0.00001) (Table 3).

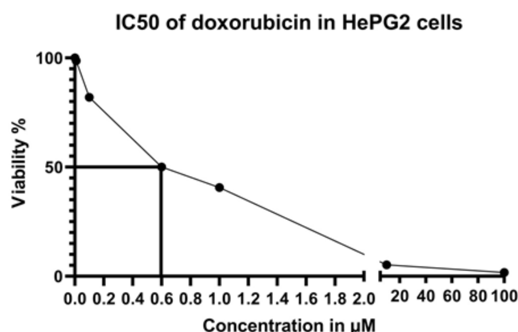

Fig. 1A: Growth inhibitory effect of doxorubicin on HepG2 cell line using different concentrations of doxorubicin showed that it's IC50. is 0.6 uM.

Table 1: Methylation of SNAI1 at different decitabine concentrations VS control untreated HEPG2.

SNAI1	Methylation	Unmethylation	P value
5 μM Control	50 99	50 1	< 0.00001
10 μM Control	89 99	11 1	0.007369
Combination Control	50 99	50 1	< 0.00001

P value is significant at < 0.05

Table 2: Methylation analysis of ECDH1 at 5 and 10 μM of decitabine VS control untreated HEPG2.

ECDH1	Methylation	Unmethylation	P value
Decitabine (5 μM) Control	89 46	11 54	< 0.00001
Decitabine (10 μM) Control	82 46	18 54	< 0.00001
Doxorubicin Control	74 46	26 54	0.000097
Combination Control	50 46	50 54	0.671125

P value is significant at < 0.05

Effect of DAC / DOX on gene expression:

The expression level for ECDH1, NCDH1, SNAI1, Twist (EMT markers) as well as Bax and Bcl2 (apoptotic markers) was assessed using qRT-PCR to elucidate the role of decitabine (DAC) on HepG2 cells. Inhibition of CDH1 induction was observed after single or combined treatment of DAC (5um/10uM) with DOX (Figure 3A). In contrast, induction of its level was reported with DOX alone. Moreover, the expression level of NCDH1 was highly decreased in all treated groups under investigation as shown in Figure 3B.

Table 3**: Methylation analysis of ECDH1 VS SNAI1 at different decitabine concentrations.**

Concentration		Methylation	Unmethylation	P value
Decitabine (5μM)	ECDH1 SNAI1	89 50	11 50	< 0.00001
Decitabine (10 μM)	ECDH1 SNAI1	82 89	18 11	0.228222
Decitabine + Doxorubicin Combination	ECDH1 SNAI1	43 50	57 50	0.394983

P value is significant at < 0.05

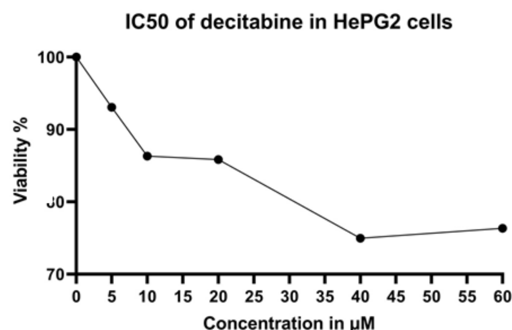

Fig. 1B: Growth inhibitory effect of decitabine on HepG2 cell line using different concentrations of decitabine showed that 5, and 10 uM Decitabine can be used as a demethylation agent without any cytotoxic effect on HepG2 cells.

As illustrated in Figure 3C, highly up-regulation of SNAI1 level was encountered in DAC (5uM) where it was 448-fold, DOX (1000-fold), DAC (5uM)/DOX (800-fold). While its level was up-regulated 26.9-fold in DAC (10uM) and 18.19-fold in DAC (10uM)/DOX combined group.

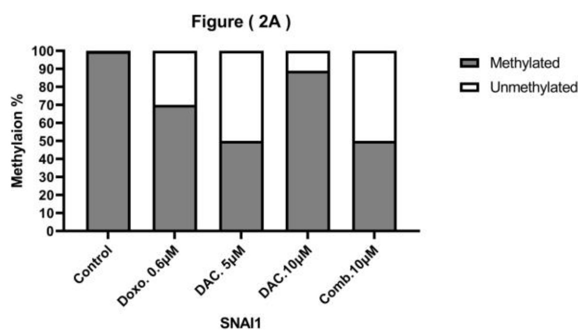

Fig. 2A: Effect of different concentrations of decitabine (DAC; 5 and 10 uM) on methylation status of SNAI1 and CDH1.

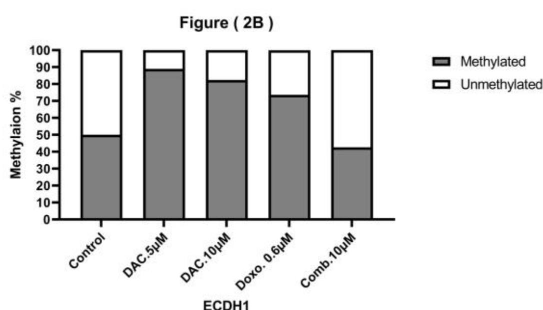

Fig. 2B: High ECDH1 methylation in HepG2 cells treated with decitabine (DAC), doxorubicin (DOXO) and the combination. (Comb)..

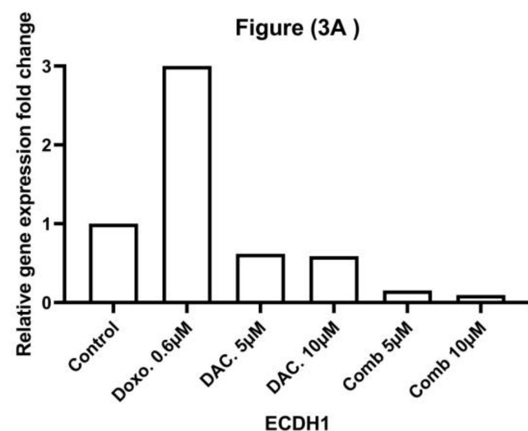

Fig. 3A: ECDH1 downregulation at 5um and 10uM concentrations of decitabine (DAC) , doxorubicin (DOXO) and DAC / DOXO Combinations (Comb) and upregulation at 0.6 uM doxorubicin concentration.

In fig 3E, Bcl2 (anti-apoptotic gene) exhibited the highest up-regulation level in both concentrations of DAC (5 uM/10 uM) as well as their combination with DOX. On the other hand, it's down regulation was reported (1.3-fold) in DOX alone. High expression of Bax (pro-apoptotic gene) was observed in DAC (5uM/10 uM) and DOX treated groups. An up-regulation of Bax was reported in DAC (10uM)/DOX group whereas it was unaffected in DAC (5uM)/DOX combined treated cells (fig 3 F).

A drastic reduction in TWIST expression level was observed in HepG2 cells treated with DAC (5uM) (588-fold) and DAC (5uM)/DOX combination (891.4-fold). A decline in its level was also reported in DAC (10uM) (32.26-fold), DOX (6.90-fold), DAC (10uM)/DOX (6.41-fold) as described in Figure 3D.

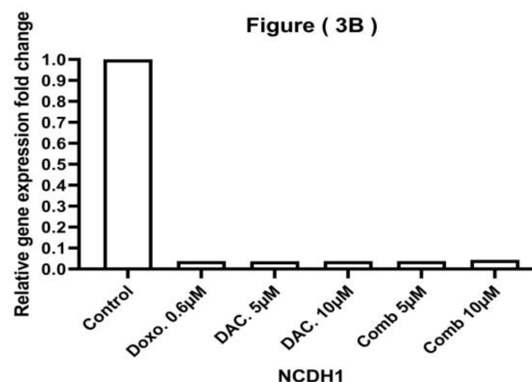

Fig. 3B: NCDH1 downregulation in decitabine (DAC), doxorubicin (DOXO) and DAC./DOX. Combination (Comb) treated cells. .

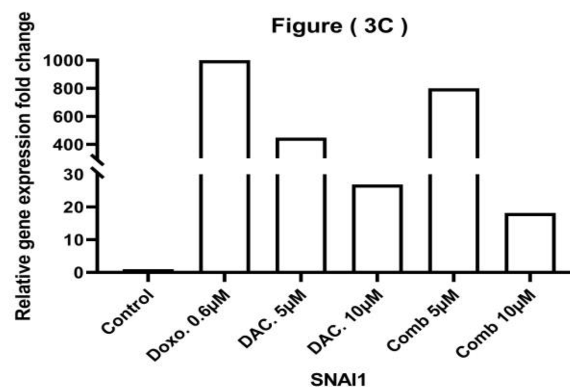

Fig. 3C: SNAI1 upregulation in decitabine (DAC.), doxorubicin (DOXO.) and DAC/DOX. Combination (Comb) treated cells.

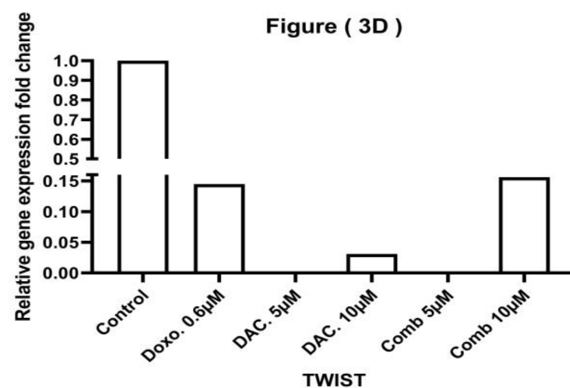

Fig. 3D: TWIST1 downregulation in decitabine (DAC), doxorubicin (DOXO) and DAC/DOXO combination (Comb) treated cells.

In Figure3E, Bcl2 (anti-apoptotic gene) exhibited the highest up-regulation level in both concentrations of DAC (5 uM/10 uM) as well as their combination with DOX. On the other hand, Bcl2 down regulation was reported (1.3-fold) in DOX alone. High expression of Bax (pro-apoptotic gene) was observed in DAC (5uM/10 uM) and DOX-treated groups. An up-regulation of Bax was reported in DAC (10uM)/DOX group whereas it was unaffected in DAC (5uM)/DOX combined treated cells (Figure 3 F).

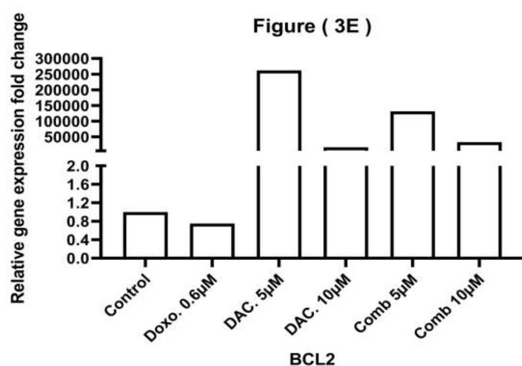

Fig 3E: Upregulation of BCL2 was observed in decitabine (DAC) 5uM, decitabine 10 uM and DAC/DOXO Combination (Comb) and downregulated 0.6 uM doxorubicin (DOXO) treated cells.

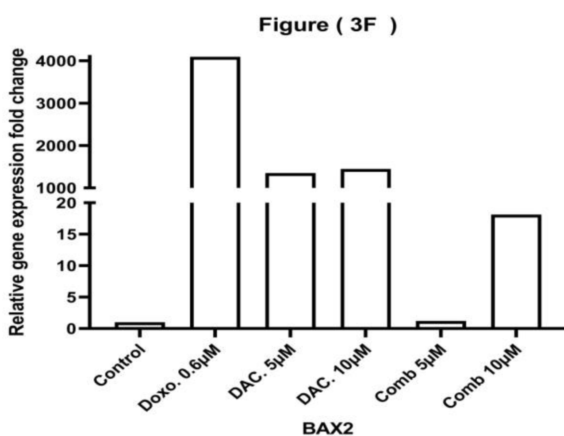

Fig. 3F: Upregulated BAX2 in decitabine (DAC; 5 and 10 uM), doxorubicin (DOXO ; 0.6 uM) and DAC/DOXO combination BAX2 expression did not affect by combination with DAC. 5 uM.

By studying the correlation between ECDH1 expression and the expression of NCDH1, SNAI1, BcL2, BAX and TWIST1, we found a highly significant correlation between ECDH1 and NCDH1 (p< 0,0001) and a near significance between ECDH1 and BcL2 (P=0.07), BAX (P=0.07) and TWIST 1(P=0.09), however, ECDH1 showed no significant correlation with SNAI1 (P 0.199). Using Chi-Square, we found a highly significant correlation between SNAI1 and BCL2 expression (P < 0.00001).

Defective apoptosis in DAC-treated cells:

In order to study the impact of Bcl2 up-regulation, we investigated the ability of DAC, DOX and their combination for induction of apoptosis in HepG2 cell line using flow cytometry assay. Cells were treated with DAC (10uM) sequentially as well as simultaneously with DOX (0.6 uM) for 72 h as compared with control untreated cells. Figure 4, our data referred to a defective apoptotic mechanism where the percent of cells in the early and late apoptosis was very low, only the cells treated with DOX for 72h showed 31.15% apoptosis and the combination of both DOX and DAC for 72h showed 15.11% apoptosis. However, a high percent of necrosis (68.15%) was detected in cells treated DOX for 72h. For DAC-treated cells, the percent of necrosis was very low (3.39%). For drug combination treated cells the percent was less than DOX treated cells (82.36%) in simultaneous and (33.07%) in a sequential application. This result indicates that the cells failed to undergo apoptosis and switched to the alternative non apoptotic cell death mechanism (necrosis) (Figure 4).

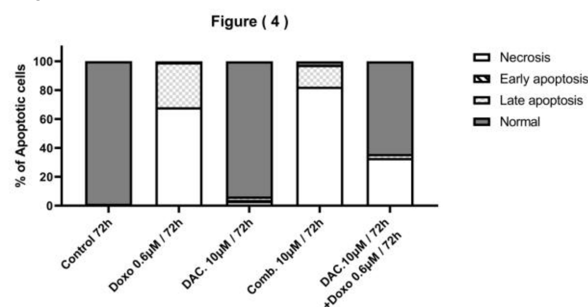

Fig. 4: Percent of apoptosis and necrotic cell death in HepG2 cells treated with decitabine (DAC) alone or combination (Comb) with doxorubicin (DOXO).

Using T-test, we studied the significance of DOX and DAC and their combination for the induction of apoptosis and necrosis. By correlating the apoptosis in DOX-treated cells with DAC and combined drug-treated cells, we found that DOX was significantly better than DAC (P = 0.00015) and both simultaneous (P = 0.00204) and sequential (P = 0.00015) drug combination for induction of apoptosis. Regarding necrosis, we found that DOX is significantly much better than DAC (P < .00001) and sequential drug combination (P = 0.00003) for induction of necrosis. These results indicate that DAC attenuates the effect of DOX for drug-induced apoptosis and necrosis.

Decitabine treated cells did not affect the cell cycle:

By studying the cell cycle phases, we found that DOX treated cells showed the highest G2 arrest after 72 h incubation (28.35%) as compared to DAC alone (17.1%) and the combination of both DAC/DOX (24.24% and 6.03). Using the T test, we found that doxorubicin alone was significantly better than decitabine alone (P= 0.0061) and sequential combination (P=0.00043) for induction of G2 arrest. However, the difference between DOX. alone and simultaneous combinations did not reach the level of significance (P= 0.114349). These data indicate that G2 cell cycle phase arrest by doxorubicin was disturbed by addition of DAC (Figure 5).

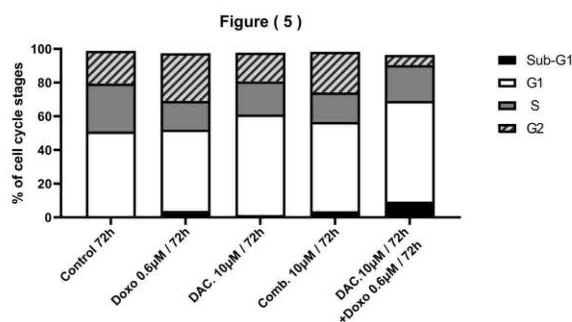

Fig. 5: Cell cycle phase analysis in HepG2 cells treated with decitabine (DAC) alone or in combination (Comb) with doxorubicin (DOXO).

Discussion:

Previous studies have demonstrated the involvement of epigenetic factors in the process of carcinogenesis and the development of cancer cells. . Promoter gene methylation is one of the epigenetic changes responsible for the carcinogenesis process in many cancers, especially liver cancer. Recent studies have proven that the process of demethylation can improve or modify gene expression in cancer cells and thus can be used as a treatment for cancer. Liver cancer is one of the most resistant cancers to chemotherapy. Therefore, we wanted to test the role of demethylating agents in improving the effectiveness of chemotherapy on liver cancer cells and to see if demethylation could enhance the response of liver cancer cells to chemotherapy. So, we studied the effect of decitabine alone and in combination with doxorubicin on the hepatocellular carcinoma cell line HePG2.

We surprisingly found an increase in methylation of ECDH1 and a decrease in methylation of SNAI1 after treatment of cells with decitabine, which indicates the possibility of activating EMT in cells, which may lead to negative effects if added to chemotherapy protocols for hepatocellular carcinoma. Methylation results were confirmed by studying the gene expression of ECDH1and SNAI1 where we found a decrease in ECDH1 expression and an increase in SNAI1 expression. In fact, previous studies assumed several explanations for the inactivation of CDH1. A recent study demonstrated that the inactivation of E cdh1 after treatment with decitabine was due to the activation of the DNA methyl transferase enzyme (18). In another study, it was shown that E- cadherin is activated after treatment of cells with decitabine at a concentration of 20 µmol and not at lower concentrations (19). This study confirms our findings, as we used concentrations of 5 and 10 µmol and did not show any effect on cadherin activation, but unfortunately activated SNAI1.

Other studies have interestingly found that E-cadherin inhibition may result from the binding of EMT transcription factors (EMT-TFs) SNAIL, SLUG (SNAI2), and TWIST to E-boxes present in the E-cadherin promoter (20). Our data indicated increased expression of SNAI1; accordingly, E-Cadherin inhibition in our study may result from the activation of the SNAI1 transcription factor, which in turn was activated by decitabine.

Therefore, we can say that according to the present findings, the sublethal concentrations of decitabine suppress the activity of the E-cadherin.

We also surprisingly found hypermethylation of ECDH1 after treatment with doxorubicin alone. Despite the increased methylation, the gene expression results of for E-cadherin showed an increase. In fact, the reason behind the increase in cadherin methylation after treatment with doxorubicin is unknown. Also, the result indicates that the increase in gene expression of E-cadherin after treatment of cells with doxorubicin is independent of its promoter methylation. Other researchers reported similar results, where they noticed that methylation is not the reason for gene expression changes in hepatoblastoma (21).

Regarding SNAI1, the present investigation showed a significant activity of this factor after adding decitabine. Other studies demonstrated the activity of SNAI1 after adding decitabine to cells in culture and also its activity in late cases of liver cancer (22). The activity of SNAI1 after decitabine treatment indicates an increase in EMT activity; especially since this activity was associated with ECDH1 inhibition (20).This may indicate a possible EMT activation in our decitabine-treated HePG2 cells, which is considered an obstacle for the use of decitabine in the treatment of HCC. Other studies have shown that the use of decitabine is not recommended for the treatment of liver cancer (23).

When we studied the anti-apoptotic factor BCL-2 in cells treated with decitabine, we found a significant increase in the expression of the gene, which indicates the possibility of cell resistance to programmed death and gives the cells a great ability

to resist chemotherapy. There are some studies that demonstrated the activity of BCL2 after decitabine treatment (24), while others demonstrated the inhibition of BCL2 and up-regulation of Bax and caspase-3 in the HCC cell line after decitabine treatment (25).

In order to determine the consequences of increased BCL2 expression on HePG2 cells, we studied the programmed cell death of cells treated with decitabine, doxorubicin, and their combination. Surprisingly, we did not find any significant apoptotic activity in cells treated with decitabine, which confirms that decitabine activates BCL2, which in turn makes the cells resistant to programmed cell death.

What confirms the validity of our results is the presence of a significantly increased necrosis in the treated cells, which indicated the inability of the cells to enter into programmed cell death as a result of the activity of BCL2 and therefore went to the alternative non apoptotic cell death mechanism the necrosis. Previous studies showed that when the apoptosis program is defective, necrotic cell death takes place as an alternative pathway (26).

We also found that doxorubicin alone induced apoptosis at a rate of about 31% and this is in line with the results of BCL2 gene expression in cells treated with doxorubicin only, where we did not find any increase in BCL2 expression. Similar results have been reported in MCF-7 where doxorubicin decreased Bcl-2 protein expression (27). Another study demonstrated that doxorubicin alters the expression pattern of BCL2 in hepatoblastoma cells (28)

This result indicates that decitabine induces the anti-apoptotic activity in HPG2 cells through activating Bcl2 and also proves that adding decitabine with doxorubicin or adding it before doxorubicin attenuates the therapeutic ability of doxorubicin.

Our study demonstrated the activation of the antiapoptotic factor BAX2 after treatment with decitabine. Other studied showed the same results (25). Although we noticed a significant activation of BAX by decitabine but this activation could not induce apoptosis in our cell model. Accordingly, decitabine decitabine- induced BAX activation is not enough to induce apoptosis especially when the anti-apoptotic factor BCL2 is active.

By comparing the results of single doxorubicin with the results of decitabine, the comparison was in favour of doxorubicin, as it was better at suppressing HPG2 cells, as evident from the results of gene expression and apoptosis. The same results apply to the combination of doxorubicin and decitabine; the results of single -agent doxorubicin were better than the combination, which gives us an indication that adding decitabine to doxorubicin may attenuate the effect of doxorubicin in HCC and limit the application of decitabine in treating liver cancer.

Conclusions

From the data of our study we may conclude that decitabine induces EMT through activation of SNAI1 and inhibition of ECDH1 in HepG2 cells. Decitabine inhibits apoptosis in HepG2 cells through activation of BCL2. Decitabine may attenuate the action of doxorubicin if the combination of both drugs was applied for the treatment of HCC. The adverse effect of decitabine may be due to its random rather than selective demethylation. The random demethylation of decitabine may be directly or indirectly activates oncogenes and this may explain the activation of SNAI1 and Bcl2 in our study. Other studies are required to study methylation status of the studied genes in HCC samples. Studying other epigenetic factors, such as the miRNA profile in HCC, may also provide more information about the epigenetic mechanisms that control transformation, metastasis, and resistance to therapy in HCC. This could aid in the development of an effective therapeutic modality for the treatment of HCC.

Conflicts of interest

"The authors declare that they have no competing interests"

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