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GFBR3 Promoter Methylation Is A Prognostic Indicator And Not Responding To Decitabine Treatment In Hepatocellular Carcinoma

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Abstract

Previous studies have demonstrated lower levels of TGFBR3 in cancer cells compared to non-cancerous cells. And the low levels are related to the clinical characteristics of the patients. However, there is no study examined the relationship between the degree of methylation of TGFBR3 and patient data in liver cancer. The aim of this study is to test the role of decitabine in demethylating TGFBR3 and the expression of miRNAs in HepG2 cells and to study the relationship between TGFBR3 methylation level and clinical data and follow-up in HCC. Five and 10 micromoles of decitabine and 0.6 micromoles of doxorubicin were used on HepG2 cells, and the degree of methylation of TGFBR3 and the expression of a group of miRNAs were assessed. The degree of methylation was studied in 14 liver cancer tissue samples and 4 adjacent non tumor tissue samples. TGFBR3 methylation levels were correlated to clinical data and patient's follow-up. TGFBR3 methylation did not change after treatment with decitabine. Decitabine upregulated MiR-10b-5p, miR-125b-5p, miR-196-5p, miR-596 in a concentration dependent manner. TGFBR3 methylation level was significantly higher in HCC than NTD (0.000456). TGFBR3 methylation level was significantly correlated to gender (0.047), grade (0.00001), LN metastasis (0.005), safety margin (0.001), AFP level (0.0003), albumin level (0.00001), platelets count (0.0005), DFS (0.00001) and OS (0.00001). Conclusion: Decitabine failed to demethylate TGFBR3 in HCC. Decitabine randomly influenced the expression of oncogenic and tumor suppressive miRNAs. TGFBR3 methylation is promising in predicting LN metastasis and survival in HCC patients.

Keywords: Decitabine, HepG2, HCC, methylation, miRNA, clinicopathologic, follow up

Introduction:

Hepatocellular carcinoma (HCC) is the third deadliest cancer in the world and one of the most aggressive tumours. [1]. HCC is more prevalent in developing nations compared to developed nations [2]. However, the disease's prevalence is increasing globally and is expected to increase in the coming years [3]

There are many causes of the disease, including aflatoxin, smoking, and alcohol, but hepatitis B and C infection is the most common cause of hepatocellular carcinoma (HCC) [4].

In Egypt, where HCV and HBV infections are prevalent, HCC is a major health concern and the second leading cause of cancer incidence and mortality in men. [1].

Surgical treatment is regarded as the most effective curative modality for HCC; however, the majority of patients do not benefit from surgery due to advanced disease stages [5]

Due to the absence of biomarkers that can detect the disease at an early stage and the resistance of HCC to existing treatments, the recurrence rate is high and survival rates are low [6]. Consequently, a greater understanding of the biology of HCC is unquestionably necessary for the development of an alternative therapeutic modality for HCC.

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HCC arise from a perturbed microenvironment in which cytokines play an essential role in orchestrating the crosstalk between tumor cells and stroma [7].

TGF is a pleiotropic cytokine from the tumor microenvironment that regulates fundamental processes, such as cell fate decisions during development, tissue homeostasis, and regeneration, and are key players in tumorigenesis [8].

TGF cytokines are activated by TGF receptor binding. There are three receptors, designated TGFBR1, TGFBR2, and TGFBR3. TGFBR3 is a proteoglycan composed of 851 amino acids with a large N-terminal extracellular domain containing at least one glycosaminoglycan attachment site, a single hydrophobic transmembrane domain, and a 41-amino acid cytoplasmic tail with no obvious signalling motif [9].

Previous research has linked decreased TGFBRIII expression to increased progression and poor prognosis in breast cancer [10], the development of pancreatic ductal adenocarcinoma [11] enhanced metastasis in renal cell carcinoma [12], advanced stage, metastasis and low overall survival in lung adenocarcinoma [13]. However, the role of TGFBR3 in HCC have not been well studied.

DNA methylation is an epigenetic mechanism that regulates gene expression and is crucial to carcinogenesis. Cancer is characterized by a deficient epigenetic mechanism, including methylation, according to recent studies [14]. Moreover, numerous studies have demonstrated that DNA methylation is responsible for the inactivation of tumor suppressor genes [15], including TGFBRIII [16].

The aim of the present work is to study the change of methylation level of TGFBRIII after treatment with the demethylating agent decitabine single and in combination with doxorubicin in HePG2 cell line. In addition, we studied TGFBRIII methylation in early versus late-stage HCC patients to study the significance of TGFBRIII promoter methylation on tumor progression.

Material and methods:

Cell line: HepG2: Hepatocellular carcinoma cell line was obtained from Nawah Scientific Inc., (Mokatam, Cairo, Egypt). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100mg/mL of streptomycin, 100units/mL of penicillin and 10% of heat-inactivated fetal bovine serum (Gibco, Life technologies Inc., UK) and maintained in humidified, 5% (v/v) CO2 atmosphere at 37 °C incubator (Thermo Scientific, City, USA).

Decitabine: Powder was obtained from DACOGEN 50mg, Janssen, Belgium and doxorubicin powder was obtained from Sigma Aldrich, USA and was dissolved in dimethyl sulfoxide (DMSO).

Human HCC Samples

Fourteen archived human HCC and 4 non-tumor distant tissues samples were isolated from patients undergoing surgery as a primary therapeutic modality during the period from 2001 to 2003 at the National Cancer Institute, Cairo University, Egypt. Department and Institutional approval was obtained. HCC diagnosis was confirmed by histopathological examination of the resected tissues by 2 independent pathologists. Clinical data as well as follow-up studies of the patients were retrospectively collected (n = 14 patients). Patients' consent was obtained before sample collection. The study was conducted according to the Declaration of Helsinki.

Cell viability assay (IC 50 determination):

Cell viability was assessed by SRB assay [17]. In brief, aliquots of 100µL cell suspension (5x10^3cells) were in 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 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 as follows: Survival fraction = O.D (treated cells)/ O.D (untreated cells), where the IC50 (the concentration of drug required to produce 50% inhibition of cell survival. Accordingly, concentration of 5, and 10µM for decitabine and 0.6 µM for doxorubicin were used for subsequent studies.

Culture media from both control and treated cells were removed and the cells were washed 3 times with 1x phosphate buffer saline (PH7.2) then were divided into 2 parts, one part was subjected to DNA extraction and methylation analysis and the other part was subjected to RNA extraction and miRNA expression analysis.

DNA extraction:

Extraction of DNA from cells and tissue samples was done using QIAamp® Fast DNA Tissue Kit catalog number 51404 (Qiagen, Germany). For each sample the following cocktail was added: 200 µl AVE, 40 µl VXL, 1 µl DX Reagent, 20 µl proteinase K, 4 µl RNase

A (100 mg/ml). samples were homogenized by vortexing for 5 min, and were then incubated in a thermomixer at 1000 rpm for 10 min at 56 C, Incubate. 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.

Promoter methylation analysis:
Promoter methylation for TGFBRIII (EPH) was studied using the Methyl Screen technology by EpiTect Methyl II Primer Assay kits (Qiagen, cat. 335002). The restriction digestions were performed using the EpiTect Methyl II DNA Restriction Kit (Qiagen,cat. 335452). Amplification was performed using an Applied Biosystem VIIA7 real-time PCR instrument (Thermo- scientific Fisher, USA). Analysis was performed using the dedicated EpiTect Methyl II PCR Array Microsoft. Excel template (www.sabiosciences.com/dna_methylation_data_analysis.php). Briefly, CT values were exported to the data analysis sheet and the percentage of promoter methylation for TGFBR3 in cells and tissue samples was automatically generated.

RNA extraction:
Isolation of RNA and miRNA from cells was done using miRNeasy Micro Kit Cat. No. / ID: 217084 (Qiagen, Germany) according to the manufacturer's recommendations. RNA concentrations and purity were determined using NanoDrop One spectrophotometer (Thermo- scientific Fisher, USA).

cDNA synthesis:
The RT2 First Strand Kit Qiagen (Cat no. 330401) was used for cDNA Synthesis from 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℃ 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℃ for 15 minutes. The reaction was stopped by heating at 95℃ for 5 minutes. 91µl of RNase-free water was added to the synthesis reaction.

Quantitative real-time PCR:
Quantification of miRNA expression treated HePG2 cell line was performed using SYBR Green RT-PCR Kit (Qiagen, Germany). The primers of target miRNAs were obtained from (Eurofins Genomics, Germany), their sequences are shown in table 1. 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.
10 µl reaction mixture per well was dispensed into MicroAmp Optical 384-well reaction plates (catalog no. 4306737 life Technology, USA). Plates were tightly sealed with MicroAmp Optical adhesive film (catalog no. 4360954 life Technology, USA). Each amplification reaction was performed in duplicate on a Via 7 real-time PCR instrument (Thermo- scientific Fisher, USA). qPCR protocol consisted of denaturation step at 95℃ for1 minutes, followed by 45 PCR amplification cycles as follows: Denaturation at 95℃ for10 sec., annealing at 60℃ for30 sec., extension at 60℃ for 30 sec. Melting curve was set for 1 cycle as follows: Denaturation at 95℃ for 15 sec, annealing at 60℃ for1 min. and extension at 95℃ for 15 sec. U6 was used as the internal control. Relative expression for each sample was calculated using 2-ΔΔCT method.

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. Data were found to be not normally distributed. Comparison between two groups was done using Mann-Whitney test (non-parametric t-test). Comparison between 3 groups was done using Kruskal-Wallis test (non-parametric ANOVA). Spearman-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:
We studied the effect of different concentrations of the demethylating agent decitabine single and in combination with doxorubicin on TGFBR3 promoter DNA methylation and on the expression of miRNA in HePG2 HCC cell line. We used 5 and 10 µM concentration of decitabine and 0.6 µM concentration of doxorubicin and correlated the results of both methylation and miRNA expression of treated cells to that of non- treated cells.
To study the influence of TGFBR3 methylation on the clinicopathological characteristics and follow up data of HCC, we studied TGFBR3 promoter methylation on 14 HCC and 4 non tumor distant (NTD) samples. The samples were taken from HCC patients after surgical resection and before receiving any type of

therapy. Methylation results were correlated to the clinicopathological and follow up data of the patients.

Effect of decitabine and doxorubicin on HepG2 cell viability:

Growth inhibitory effect of doxorubicin (DOX) and decitabine (DAC) on HepG2 cell line was assessed using (SRB) assay as illustrated in fig 1a. DOX showed 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 poor cytotoxic effect at low doses as presented in fig 1b. Based on the present data, low doses of DAC (5, and 10 uM) can be used in demethylation without any cytotoxic effect on HepG2 cell in the subsequent experiments.

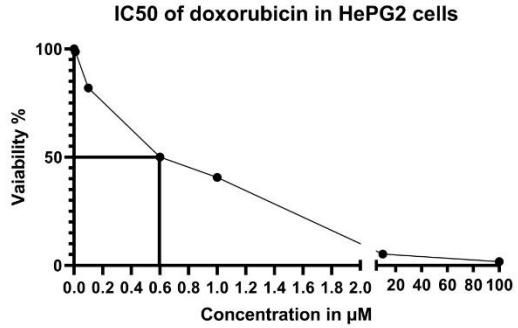

Fig. 1a: **Growth inhibitory effect of doxorubicin on HepG2 cell line using different concentrations of DOX showed that the IC50 of doxorubicin. is 0.6 uM**

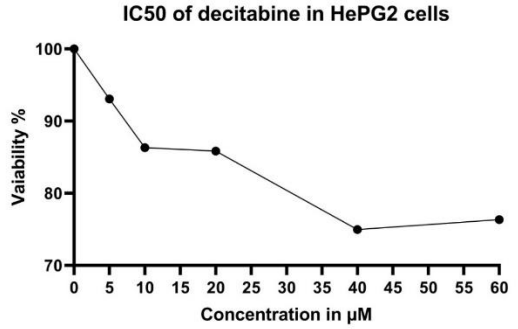

Fig. 1b**: Growth inhibitory effect of decitabine on HepG2 cell line using different concentrations of decitabine showed that 5, and 10 uM decitabine can be used for demethylation agent without any cytotoxic effect on HepG2 cells.**

Decitabine and doxorubicin treatments did not change TGFBR3 promoter methylation in HePG2 cells:

Application of Single doses of decitabine (5 and 10 Mm) and doxorubicin (0.6Mm) in addition to the combination of both drugs showed no effect on promoter methylation of TGFBR3 in HepG2 cells, where the median methylation percent remained 50% in both treated and untreated cells fig. 2.

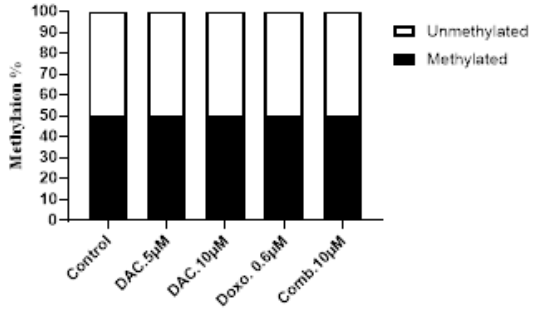

Figure 2: **Methylation profile of decitabine and doxorubicin treated HepG2 cells.**

Decitabine changed the expression of miRNA in HePG2 cells in a concentration dependent manner:

Gene expression of 12 MicroRNAs responsible for several processes was studied in HPG2 cells after treatment with doxorubicin and decitabine and their combination. Only 4 miRNAs (MiR-10b-5p, miR-125b-5p, miR-196-5p, miR-596) were selected, as the remaining 8 did not show any expression.

As shown in fig. 3, miR-10b-5p expression showed upregulation at decitabine concentration of 5 µM and downregulation at concentration of 10 µM. However, the combination of doxorubicin and decitabine showed miR-10b-5p upregulation at concentrations of 5 µM and 10 µM.

MiR-125b-5p expression showed up regulation at concentration of 10 µM of both decitabine and the combination.

MiR-196-5p expression showed upregulation at decitabine concentration of 5 and 10 µM and combination of 10 µM. However, miR-196-5p showed downregulation at combination of 5 µM.

MiR-596 expression showed upregulation at decitabine concentration of 10 µM and downregulation at decitabine concentration of 5 µM. The combination of both drugs showed MiR-596 upregulation at 5 and 10 µM.

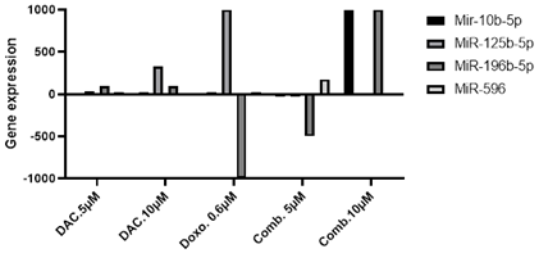

Figure 3: **MiRNA expression profile in decitabine and doxorubicin HepG2 treated cells.**

The 4 studied miRNAs are significantly correlated to each other:

Using the Chi-square test we found a highly significant correlation between the expressions of the 4 miRNAs (< 0.00001). This relation may indicate a common mechanism between these miRNAs (Tables 1a, ab, ac).

Table1a: Correlation between mir-10b-5p versus miR-125b-5p, miR-196b-5p and miR-596 in Decitabine. and Doxorubicin treated HepG2 cells.

	DAC. 5 µM	DAC. 10 µM	Doxorubicin	Comb. 5	Comb. 10	p value
MiR-10b-5p	17	-3	-2	6	1000	
MiR-125b-5p	-1	325	1000	6	7	< 0.00001
MiR-196b-5p	100	100	-1000	-500	1000	< 0.00001
MiR-596	-6	10	-2	176	7	< 0.00001

Table 1b: Correlation between miR-125b-5p versus miR-196b-5p and miR-596 in Decitabine and Doxorubicin treated HepG2 cells.

	DAC. 5 µM	DAC. 10 µM	Doxorubicin	Comb. 5	Comb. 10	p value
MiR-125b-5p	-1	325	1000	6	7	
MiR-196b-5p	100	100	-1000	-500	1000	< 0.00001
MiR-596	-6	10	-2	176	7	< 0.00001

Table 1c: Correlation between miR-196b-5p and miR-596 in Decitabine and Doxorubicin treated HepG2 cells.

	DAC. 5 µM	DAC. 10 µM	Doxorubicin	Comb. 5	Comb. 10	p value
MiR-196b-5p	100	100	-1000	-500	1000	< 0.00001
MiR-596	-6	10	-2	176	7	< 0.00001

Clinicopathological characteristics of the studied HCC samples:

Samples were collected from 9 males (64.3%) and 5 females (35.7 %) with a media age of 53 years (Range 38 min. 30 max.68). Eight samples were G2 (57.1 %), 2 were G3 (14.3%) and 4 samples were missing (28.6%). Two patients had +ve lymph node (14.3 %), 8 patients had –ve lymph node (57.1 %) and 4 were missing (28.6%). Two patients had +ve safety margin (14.3 %). 11 had –ve safety margin (78.6%) and 1 was missing (7.1%). Six patients had vascular invasion (42.9 %), 2 patients had no vascular invasion (14.2%) and 6 patients were missing (42.9 %). Eleven patients had cirrhosis (84.6 %), 1 patient had no cirrhosis (7.7%) and 1 was missing (7.7%). The median AFP was (28), the median GOT was (60), the median GPT was (77.5), the median ALB was (4.15), the median leucocytic count was (5.37) and the median platelets count was (161). Six patients had recurrent disease (42.9%), 5 had no recurrence (35.7%) and 3 were missing (21.4%). Four patients had metastasis (28.6%), 6 were non-metastatic (42.8%) and 4 were missing (28.6%). The median disease-free survival (DF S) was 9.5 months and the medial overall survival was 12.5 months (Table 2).

Table 2: Clinicopathological characteristics and follow up data of the studied HCC patients.

Patient Characteristics		Number	Percent
Median Age	53 (Range 38 min. 30 max.68). SD (10.62)	14	100%
Sex	Male	9	64.3%
	Female	5	35.7 %
Grade	II	8	57.1 %
	III	2	14.3%
	Missing	4	28.6%
L.N	Yes	2	14.3 %
	No	8	57.1 %
	Missing	4	28.6%
Safety margin	Yes	2	14.3 %
	No	11	78.6%
	Missing	1	7.1%
Vascular invasion	Positive	6	42.9 %
	Negative	2	14.2%
	Missing	6	42.9%
Cirrhosis	Positive	12	85.7 %

	Negative	1	7.15 %
	Missing	1	7.15%
Median AFP	28 (1.9 – 27389) SD: 8183.4563	11	78.5 %
	Missing	3	21.5%
Median GOT	60 (7– 182) SD: 60.765	13	93%
	Missing	1	7%
Median GPT	77.5 (16 – 409) SD: 104.807	12	85.7%
	Missing	2	14.3%
Median Albumin	4.15 (2.5 – 6) SD: 1.19	8	57%
	Missing	6	43%
TLC	5.37 (2.99– 18.5) SD: 3.893	13	93%
	Missing	1	7%
PLT	161 (64 – 661) 159.862	13	93%
	Missing	1	7%
Metastasis	Positive	4	28.6%
	Negative	6	42.8%
	Missing	4	28.6%
Recurrence	Yes	6	42.9%
	No	5	35.7%
	Missing	3	21.4%
DFS	9.5 months (0.33 - 16.43) SD: 5.497	6	43%
	Missing	8	57%
OS	12.53 months (0.13 - 31.63) SD: 11.347 Missing	12	85.7%
		2	14.3%

TGFBR3 methylation is higher in HCC than NTD samples:

For HCC, the median methylation percent was 50% and the median unmethylation percent was 50%. For NTD samples, the medial methylation was 25.2% and the median unmethylation was 74.8% (Table 3). Using Chi square to correlate the methylation between HCC and NTD samples. We found a significantly negative correlation between the methylation of HCC and NTD samples (0.000456) (Fig.4)

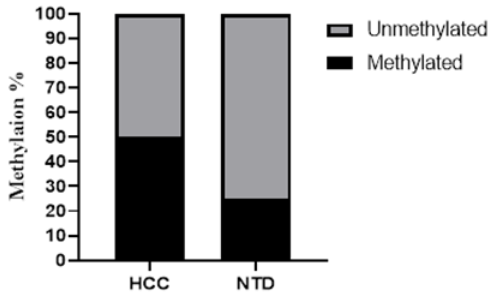

Figure 4: Methylation status of TGFBR3 promoter for HCC versus NTD tissue samples.

Correlation between TGRBR3 promoter methylation level and the clinicopathological and follow up data of HCC patients:

By correlating TGFBR3 methylation to the clinical and follow up data of the patients we found a significant correlation between methylation level and patients gender (p 0.047), tumor grade (p 0.0000), lymph node status (p 0 .005), safety margin (p 0.0011), AFP level (p 0.0003), albumin level ($p < 0.00001$), platelets count (p 0.0005), DFS (P 0.00001) and OS ($P < 0.00001$). However, TGFBR3 methylation did not show any significance with recurrence (p 0.392) or metastasis (p 0.197) (Table4).

Previous studies indicated that decreased expression of TGFBR3 is associated with low survival in cancer [10]. We tried to demethylate the promoter of TGFBR3 in HepG2 cells by application of different concentrations of decitabine in an attempt to enhance the expression of TGFBR3. Our results indicated that different concentrations of decitabine failed to change the methylation of TGFBR3 in HepG2 cell line. Recent studies found different mechanisms for decitabine resistance, of these mechanisms are adaptive responses of the pyrimidine metabolism network [18], or mutations in the dck gene [19]

Table 3: Correlation between TGFBR3 promoter methylation of HCC samples and NTD samples.

	Number	Median Methylation	Median Unmethylation	P value
HCC	**14 (0.11-97.78) SD: 35.09**	**50 (0.11-97.78) SD: 35.09**	**50**	**0.000456**
NTD	**4**	**25.2(0.05-93.6) SD: 45.005**	**74.8**	

Table 4: Correlation between TGRBR3 promoter methylation and the clinicopathological and follow up data of the studied HCC patients.

Data	TGFBR3		P value
	Median Meth. %	Median Unmeth. %	
Age	39	61	
≥ 53	49	51	0.1543
< 53			
Gender	40	60	
M	54	46	0.047315*
F			
Grade	24	76	
G2	84	16	
G3			0.00001***
L.N	38	62	
+Ve	40	60	
-Ve			0.005991**
Safety margine	25	75	
+Ve	47	53	0.001192**
-Ve			
Alfa feto-protein	31	69	
≥ 28	56	44	
< 28			0.000363***
GOT	37	63	
≥60	51	49	0.064046
< 60			
GPT	41	59	
≥77.5	38	62	0.772357
< 77.5			
Albumin	21	79	
≥4.15	53	47	
< 4.15			< 0.00001***
TLC	45	55	
≥5.37	42	58	0 .775442
< 5.37			
PL	55	45	
≥161	30	70	0.000597***
< 161			
Recurrence	40	60	
Y	47	53	0 .392114
N			
Metastasis	37	63	
Y	47	53	0.197258
N			
DFS	73	27	
≥ 9.5	26	74	0.00001***
< 9.5			
OS	58	42	< 0.00001***
≥ 12.53	24	76	
< 12.53			

Discussion:

This result indicates that decitabine failed to demethylate and accordingly failed to activate TGFBR3 in HCC. On the other hand, decitabine influenced the expression of the 4 studied miRNAs, miR-10b-5p, miR-125b-5p, miR-196b-5p and miR-

596. MiR-10b-5p in a concentration dependent manner where 5 µM decitabine upregulated its expression while 10 µM downregulated its expression. Recent studies indicated that miR-10b-5p inhibit the proliferation and invasion of hepatic carcinoma cells [20] and is considered as a promising biomarker for early-stage HCC [21]. On the other hand, down regulation of miR-10b-5p is associated with liver metastasis in colorectal cancer [22]. According to our results the low concentration of decitabine may activate miR-10b-5P which may disturb tumor progression. The other miRNA marker miR-125b-5p was up-regulated after treatment with decitabine and the combination of both decitabine and doxorubicin. Studies have demonstrated a tumor suppressive role for miR-125b-5p [23] [24], this is in addition to its effective role in increasing the sensitivity of cancer cells to chemotherapy [25]. In HCC, miR-125b-5P was found to inhibit the proliferation and invasion of cancer cells. [26]. Our result indicates a positive effect of decitabine on the expression of miR-125b-5p and accordingly, it has an inhibitory effect on the process of proliferation and invasion of cancer cells. The third miRNA is the oncogenic miR-196b-5p, our results showed up regulation of this miRNA by decitabine. miR-196b-5p was found to be upregulated in different types of cancers including colorectal [27], lung adenocarcinoma [28] and HCC [29] In addition, it was involved in chemotherapeutic resistance [30]. This is considered one of the undesirable effects of decitabine, as although it stimulates microRNAs that inhibit carcinogenesis, it also stimulates others that stimulate cancerous growth and resistance to anti-cancer treatments. As for mir-569, our results showed a decrease in its expression when using a dose of 5 µM of decitabine, while a dose of 10 µM and the combination of dec./dox together highly increased its expression. Recent studies indicated a tumor suppressive role of miR-569 in different types of cancers including lung cancer [31], pancreatic cancer [32], and aggressiveness of epithelial cancers [33].

Our results showed that decitabine did not affect the methylation of tgfbr3 in HepG2 cells. On the contrary, it affected the expression of 4 miRNAs, three of which are tumor suppressors and one that is carcinogenic. Although the effect on miRNA was concentration dependent, these results indicated that the effect of decitabine was random rather than selective as it activated both oncogenes and tumor suppressors. Previous studies have shown that decitabine activated carcinogenic factors such as SNAI1 [34].

Therefore, great care must be taken before using decitabine in the treatment of liver cancer. Also, more studies should be done to draw conclusive results regarding the effect of decitabine on the methylation process in liver cancer cells.

Regarding HCC samples, we found significantly higher TGFBR3 methylation than non-tumor distant tissue samples. This result support the previous studied which indicated methylation of TGFBR3 in cancer [35]. By correlating the methylation of TGFBR3 to the clinicopathological and follow up data we found a significant correlation with the clinical and follow up data including overall survival and disease free survival. Recent studies found that low level of TGFBR3 in HCC is associated with bad prognosis [36], metastasis [37] grade [38] and alfa fetoprotein level [38]. However, ectopic expression of TGFBR3 prevents lymph node metastasis in head and nick cancer [39].

Although recent results have shown that low levels of TGFBR3 affect clinical information and follow-up of patients, these studies did not establish the cause of low levels of TGFBR3. Our study has shown that methylation of TGFBR3 has a strong relationship with clinical information and patient follow-up, and the degree of methylation of TGFBR3 may be the reason for its low level in liver cancer. This explanation seems logic because the degree of methylation affects gene expression.

Although our study proved the ineffectiveness of decitabine in demethylating TGFBR3 in HepG2 cells, the level of methylation in TGFBR3 is considered an important factor related to several clinical factors and also prediction of survival in HCC patients.

Therefore, we recommend the use of TGFBR3 methylation level in predicting survival of patients with HCC. We also recommend the use of alternative drugs or treatment modalities that can influence the demethylation of TGFBR3 in HCC where the demethylation of this gene and thus increasing its expression level will have a positive and effective impact on the treatment of HCC.

Although the number of samples in our study is relatively low, but the preliminary results of our study offers a promising data regarding the clinical and prognostic significance of TGFBR3 methylation in HCC.

Conclusion:

Decitabine failed to demethylate TGFBR3 in HCC. Decitabine influenced the expression of oncogenic and tumor suppressive miRNAs in a concentration dependent manner. TGFBR3 methylation is promising in predicting LN metastasis and survival in HCC patients.

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