

MicroRNA-26a systemic administration attenuates tumor formation in hepatocellular carcinoma mouse model

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Abstract: MicroRNA (miRNA)-26a is one of the tumor suppressor genes that has been down regulated during the development of hepatocellular carcinoma (HCC). This work was conducted to evaluate the possible preventive effect of exogenous miRNA-26a administration on diethylnitrosamine (DEN)-mediated HCC. Balb/C mice were intraperitoneally injected with saline (Normal group), DEN (HCC group) or miRNA-26a (HCC+miRNA-26a group). On week 8, 12, 16 and 20, the concentrations of alpha-fetoprotein (AFP), des-gamma carboxyprothrombin (DCP), the levels of helper T cells-associated cytokines, and the vascular endothelial growth factor (VEGF), were measured. Flow cytometry determined the frequencies of regulatory T (Treg) cells. The concentrations of AFP, DCP and VEGF, as well as the frequency of Treg cells showed significantly lower values following miRNA-26a administration than in HCC group. miRNA-26a administration has reduced the levels of IL (interleukin)-2 and TNF (tumor necrosis factor)- α , in contrast, IL-10 level was markedly elevated in comparison to HCC model at all experimental time points. The restore of miRNA-26a function significantly ($P < 0.001$) down regulated the expression levels of survivin & caspase-3 compared to HCC group. The obtained data introduce an evidence for the suppressive impact of miRNA-26a on liver tumor formation and its possible manipulation as a therapeutic design for HCC.

Keywords: HCC, miRNA-26a, DEN, tumor markers, regulatory T cells, cytokines, survivin, caspase-3, VEGF.

INTRODUCTION

Hepatocellular carcinoma (HCC) is an extremely aggressive type of malignancy that is frequently grown in many countries across the globe (Lovet *et al.*, 2003). It is the third most prominent cause of cancer-related death in the world (Bertuccio *et al.*, 2017). HCC treatment involves many options such as operational removal, transplantation, radiotherapy, and chemotherapy (Bruix and Sherman, 2011). Drug resistance, recurrence, and poor prognosis among these therapeutic approaches of HCC are still a challenge, particularly in advanced stages (Jemal *et al.*, 2011). Therefore, it is important to define new therapeutic targets of HCC.

MicroRNAs (miRNAs) are a class of short single stranded noncoding RNAs, varying in length between 20 and 25 nucleotides. miRNAs negatively regulate the level of post-transcriptional gene expression by binding to the 3'-untranslated region of messenger RNAs that lead to interfering with their translation or the decomposition of formed transcripts (Bartel, 2009). According to these functions, many biological processes are controlled by miRNAs like cellular development, proliferation, apoptosis, metabolism and tumorigenesis (Lin and Gregory, 2015). The CD4⁺ T helper (Th) cells are a subpopulation of T cells that perform a fundamental role

in the creation of adaptive immune responses during tumor growth (Robinson *et al.*, 2016). Several kinds of miRNAs are involved in the differentiation of Th cells as well as regulation of their production of cytokines, chemokines, and growth factors (Jindra *et al.*, 2010; Sethi *et al.*, 2013). Aberrant expression of miRNAs is commonly evidenced in several cancers like HCC and the essential role of miRNAs in tumor development, angiogenesis, invasion, and metastasis has been addressed (Shyu *et al.*, 2016). Charged composition and low weight of miRNAs make them an effective tool and attractive option for the advancement of clinical cancer therapy (Chen *et al.*, 2015).

There is a widespread use of miRNAs in clinical practices as diagnostic and therapeutic tools (Ni and Leng, 2015). The theory of miRNA treatment is known by recovering the expression failure through exogenous miRNA or even its inhibition via miRNA antagonists. miRNA-26a is a member of miRNA-26 family and recognized in HCC as a possible tumor inhibitor. Several studies have implied the down regulation of miRNA-26a expression in bladder cancer (Miyamoto *et al.*, 2016), gallbladder cancer (Zhou *et al.*, 2014) and breast cancer (Gao *et al.*, 2013). It inhibits tumor growth indirectly in HCC by preventing angiogenesis and intratumoral infiltration of macrophages (Chai *et al.*, 2015). A clinical investigation of more than 200 patients showed that the high miRNA-26 expression has a good correlation with improved overall survival (Ji

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et al., 2009). Furthermore, the miRNA-26a plasma levels were utilized as a possible pronounced biomarker for predictive intervention during the treatment of HCC patients (Kim *et al.*, 2018).

Systemic delivery of miRNA is one of the applicable methods in which miRNA needs to pass through the blood flow to the targeted tissue. This research investigation was conducted to compensate for the loss of miRNA-26a expression that was down-regulated during liver tumor progression via intravenous administration of its synthetic form into the DEN-induced HCC mouse model. The efficacy of this replacement treatment strategy was monitored during phases of liver tumor formation as a protective tool against HCC development during the determined time points of disease follow-up. The impact of miRNA-26a administration on the frequency of T-regulatory (Treg) cells and Th-related immune responses was estimated.

MATERIALS AND METHODS

Animals

Male Balb/C mice of two weeks old were kindly provided by Theodor Bilharz Research Institute (TBRI) (Giza, Egypt). Animals were maintained under 12h of light/dark, fed on the standard pellets of diet and provided with free access to water. All experimental procedures applied to animals were accepted by the ethics committee of TBRI (No. 23/03/15) and carried out in compliance with the Guide for the Care and Use of Laboratory Animals, 8th edition.

Induction of hepatocellular carcinoma

In mice, tumors were produced by intra-peritoneally (i.p) injected DEN (Sigma, Aldrich), diluted in a phosphate buffered saline (PBS). The dosage of 50mg/kg body/weight was administered weekly for 16 weeks by using a 29 G syringe, resulting in DNA damage and cellular changes (Mansy *et al.*, 2017).

Design of miRNA-26a

miRNA-26a was manufactured as the following sequence of miR-26a precursor: 'tcgaggatccgtgatcacaaaggtcccagggtctgggtcagaaaattctctccgaggggaatgaagccacaggagccaagagcaggagaccaaggccctggcgaaggccgtggcctcgttcaagtaatccaggatagcgtgtgcaggtcccaatgggcctattcttggttacttgcacggggacgcggcctggacgcgcgcatccgggtcagacccccctctctgccagaggcaccacacacagagttcacaatcagtcctctgcccttgcattgtagcaagctagctcga' (Genscript, company, USA).

Experimental design

A hundred and twenty mice were classified into three main groups (40 mice/each); Control group (Normal): injected i.p. with PBS as a vehicle control; mice in HCC group received i.p. injection with DEN; mice in HCC+miRNA-26a group were injected intravenously

only once via tail vein with 100µl of synthetic miRNA-26a for each mouse after one-week post-injection of DEN.

Following DEN administration on weeks 8, 12, 16 and 20, 10 animals in each group were subjected to blood sampling from retro-orbital sinus under slight anesthesia using 2% -3% of isoflurane. The mice were euthanized by cervical dislocation and were dissected to harvest livers, and then fixed for histological examination in 10% buffered formalin.

Measurement of tumor markers

Serum concentration of alpha-fetoprotein (AFP) was quantified by Quantikine® enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., USA) and expressed as ng/ml. Quantitative determination of des-gamma carboxyprothrombin (DCP) concentration was measured by a sandwich ELISA (Bioassay Technology laboratory, Shanghai, China) and defined as mAU/ml. All steps were performed according to the manufacturing instructions.

Cytokine assays

Cytokine levels of tumor necrosis factor (TNF)-α, interleukin (IL)-2 and IL-10 were quantitatively measured by ELISA kits (BioLegend, MAX™, Inc., San Diego, CA, USA) according to manufacturing instructions. Unknown cytokine concentration was determined by a standard curve fitting data and represented as pg/ml.

Flow cytometry detection of CD4+CD25+ regulatory T cells

The blood was collected and placed into a tube with an EDTA anticoagulant within 6 hours. The cells were treated with anti-CD4 monoclonal antibodies labeled with PE (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and anti-CD25 monoclonal antibody labeled with FITC (BD Pharmingen™). A minimum of 10µl from each mAb were applied to one hundred microliters of the blood and incubated at room temperature for 10min. Cells were subsequently lysed using 500µl of OptiLyse C solution for further 10min after adding 500µl of PBS solution. Control samples were handled in the same way without mAbs to obtain the main cell population's basic histogram and adjust the area of autofluorescence. The ready sample has investigated by Coulter Epics XL (Beckman Coulter Inc., Fullerton, California, USA).

Estimation of survivin and casapase-3 by real time polymerase chain reaction

RNA extraction and cDNA synthesis

Total RNA was obtained from liver specimens by RNeasy extraction kit (Qiagen). RNA concentration was quantified by using spectrophotometer (NanoDrop2000). Reverse transcription was then achieved to produce cDNA corresponded to mRNA that was isolated from

specimens. The cDNA reverse transcription kit was used to generate cDNA by Thermocycler (Germany), under; 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and then at 4°C the reaction was inhibited.

Quantitative reverse transcription real time PCR (qRT-PCR)

Amplification of cDNA was carried out by SYBR Green I (Thermo Scientific) and real time (RT)-PCR 7500 (Life Technologies, Applied Biosystems, Foster City, CA, USA). In each experiment, β -actin for endogenous regulation was used to normalize the housekeeping gene. Sequence of survivin forward primer was F-5'-TGCCCCGACGTTGCC-3' and survivin reverse primer was R -5'-CAGTTCTTGAATGTAGAGATGCGGT-3'CTCAGTGG-3'. Caspase-3 forward primer was F-5'-TTCAGAGGGGATCGTTGTAGAAGTC-3' and caspase-3 reverse primer was R -5'-CAAGCTTGTCGCATACTGTTTCAG-3'. β -actin forward primer was F-5'-GGCATCCTGACCCTGAAGTA-3' and β -actin reverse primer was R-5'-GGGGTGTGAAGGTCTCAA-3'. For each qRT-PCR reaction, 2 μ l of the cDNA products, 12.5 μ l of RT SYBR Green ROX qPCR master mix (Qiagen), 1 μ l each of the forward / reverse, 8.5 μ l free RNAase water, were mixed reaching 25 μ l final reaction volume. The relative expression of these markers was quantified by using of the comparative threshold cycle (CT) method.

Estimation of vascular endothelial growth factor

Quantikine® ELISA (R&D Systems, inc., Minneapolis, USA) kit provided a quantitative measurement of mouse level of VEGF in serum samples. Every step followed the instructions of the manufacturer.

STATISTICAL ANALYSIS

One Way ANOVA (parametric) or Kruskal -Wallis (non-parametric) were used to investigate the significance of experimental periods on the estimated parameters. Duncan's test to homogeneity or Man -Whitney was used to compare between each two dependent variables. Data were represented as a mean of 10 mice $\pm$ standard error of the mean (SEM). Data exhibited a significant difference at $P < 0.05$. Regression analysis and correlation coefficient were used to fit the relationship among the measured parameters. Percentage of the change was calculated in the HCC group versus control group, in treated group versus HCC group. The statistical analysis was achieved by the Social Sciences Statistics Software (SPSS) version 24.6.

RESULTS

Tumor markers

The HCC group displayed a significant elevation in the concentrations of AFP (fig. 1A) and DCP (fig. 1B) compared to the normal group at all the same respective

time points ($P < 0.001$), and reached the maximum increase (419%) on week 16. At all experimental time points, the HCC group that administered with miRNA-26a implied a significant reduction in the serum concentrations of AFP and DCP compared to the HCC group, the highest reduction in their levels was -66.4% and -63.5% in weeks 16 and 12, respectively.

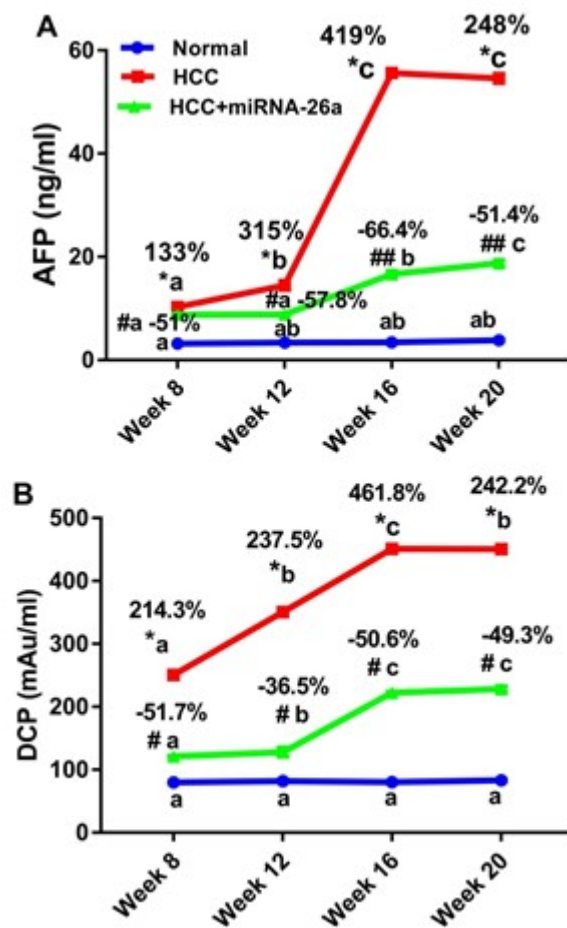


Fig. 1: Tumor markers. Concentrations of (A) AFP (ng/ml) and (B) DCP (mAu/ml) in sera of control mice (normal group) and those injected with DEN (HCC group) and DEN plus miRNA26a (treated group) during the experimental periods. Data were mean of 10 mice $\pm$ SEM. Values with the same superscript letters in the group are insignificantly differ, ($P \geq 0.05$) whereas others (significant, $P < 0.05$). * $P < 0.001$ significant difference vs. normal group # $P < 0.05$, # $P < 0.001$ significant difference versus HCC group.

Histological assessment

The control group showed normal appearance of a radial arrangement of hepatocytes rays around the central vein (long arrows) and were separated by sinusoids (short arrows) (fig. 2a). After 20 weeks of follow up, the liver sections of the HCC group, in grade 2, were structurally disturbed in the case of malignant hepatic cells, including nuclear atypical and cholestasis (fig. 2b). Interestingly, administrating of miRNA-26a revealed a regression of

malignant damage (fig. 2c). Most of lobular architecture was restored, but few foci of cholestasis were still resistant (fig. 2d).

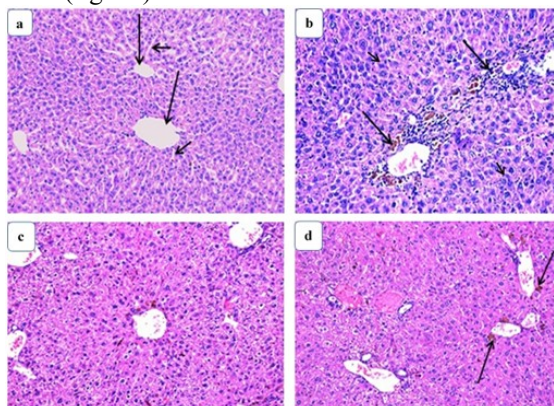


Fig. 2: Histological features of liver tissue after 20 weeks. Specimens stained with H&E showed: (a) normal architecture (normal group) of hepatocytes; (b) disturbed architecture; malignant hepatic cells with nuclear atypia (short arrows) and cholestasis; indicated by brownish pigment (long arrows) (HCC group); (c) regression of malignant change with almost restoration of lobular architecture; (d) show regression of malignant change, with disturbed architecture and resistant few foci of cholestasis; indicated by brownish pigment (arrows) (HCC+miRNA-26a group). Magnification of X200.

Expression of CD4⁺ CD25⁺ Treg cells

The frequencies of Treg cells in the HCC group were significantly higher ($P<0.001$) than those in the control group during all weeks of investigation, reaching the maximum increase (349.4%) after 16 weeks. The frequency of Treg cells in the HCC+miRNA-26a group was markedly decreased ($P<0.001$) in comparison to the HCC mice at all periods of follow up, the highest decrease (-74.6%) was detected after 16 weeks (fig. 3A).

T helper 1-related cytokines

Levels of TNF- α and IL-2 were quantified by ELISA and illustrated in fig. 3B&C respectively. Both cytokines exhibited significantly higher levels in the HCC group compared to the control mice ($P<0.001$) during all investigated time points. The highest elevation in TNF- α level was 253.1% after 20 weeks, while IL-2 was 736.9% after 16 weeks in comparison to the control group. miRNA-26a administration (HCC+miRNA-26 group) suppressed the TNF- α and IL-2 levels significantly ($P<0.001$) in comparison to the HCC group at all investigated weeks. The maximum decline was -44.1% in the 16th week and -41.2% in the 20th week for TNF- α and IL-2, respectively.

T helper 2-related cytokines

IL-10 levels in the HCC group were significantly higher across all weeks of follow-up, with the maximum rise (605.4%) in the HCC group at week 16. Interestingly, miRNA-26a administration resulted in a dramatic

elevation in the levels of IL-10 in comparison to the HCC group ($P<0.001$) during all weeks, with the maximum increase (136.5%) after 12 weeks (fig. 3D).

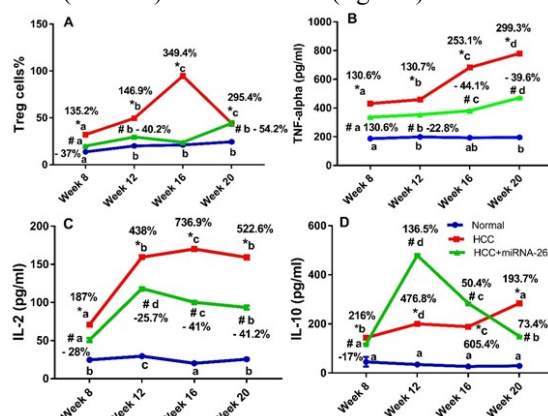


Fig. 3: T helper cells and frequency of Treg cells. (A) The frequency of CD4⁺CD25⁺ Treg cells; the levels of TNF- α (B), IL-2 (C) and IL-10 (D) in sera of normal mice (control group) and those injected with DEN (HCC group) and DEN plus miRNA26a (treated group) during the experimental periods. Values are the mean of 10 mice $\pm$ standard error of the mean (SEM). Values with the same superscript letters in the group insignificantly differ ($P\geq0.05$) whereas others significant ($P<0.05$). * $P<0.001$ a significant difference vs. control group; # $P<0.001$ a significant difference vs. HCC group.

Apoptotic markers

The expression levels of survivin in the HCC group were significantly higher than in the normal group throughout the experiment ($P<0.001$) in liver tissues; the maximal increase was 419% after 16 weeks (fig. 4A). In comparison to control group, caspase-3 has been increased significantly in the HCC group; the maximal increase was 299% after 12 weeks (fig 4B). Simultaneous administration of miRNA-26a (HCC+miRNA-26a group) demonstrated a significant reduction in the expression levels of survivin and caspase-3 in comparison to the HCC group at all weeks ($P<0.001$), this down regulation implied the highest decrease (-66.4%) after 16 weeks for survivin and 12 weeks for caspase-3 (-58.6%) as compared to the HCC group.

Estimation of VEGF

Following DEN exposure, the HCC group showed a substantial rise following 12, 16 and 20 weeks in comparison to control mice ($P<0.001$), correlated to the maximum increase (324.2%) at the 20th week. HCC+miRNA-26a group recorded a significant decline in VEGF levels in comparison to the HCC group after 12, 16 and 20 weeks ($P<0.001$) with the highest decrease (-74.5%) in the 20th week (fig. 4C).

Correlation between the measured parameters

After 20 weeks of follow up in HCC+miRNA-26a group, the frequency of Treg cells showed a strong positive

correlation with the levels of IL-2 ($r=0.892$, $P=0.001$) (fig. 5A). Concentrations of DCP were substantially positive correlated to VEGF levels ($r=0.853$, $P=0.002$) (fig. 5B) but have shown a marked reverse dependence on relative expression of survivin ($r=-0.711$, $P=0.021$) (fig. 5C).

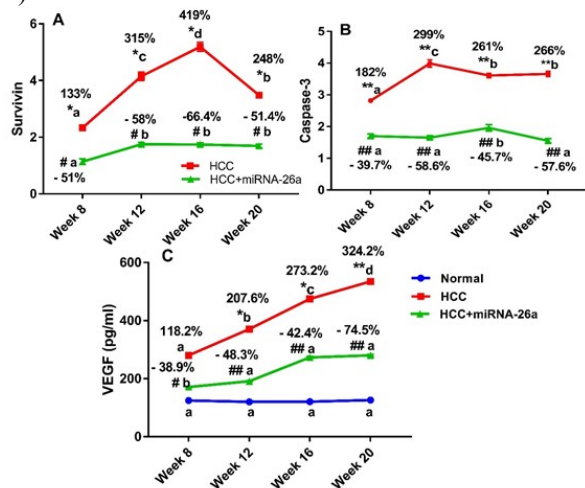


Fig. 4: Expression of apoptotic and angiogenesis markers. The expression levels of (A) survivin and (B) caspase-3 in the liver mice as well as the serum level of (C) VEGF in control mice (normal group) and those injected with DEN (HCC group) and DEN plus miRNA26a (treated group) during the experimental periods. Data were expressed as a mean of 10 mice $\pm$ standard error of the mean (SEM). Values with the same superscript letters in the group insignificantly differ ($P \geq 0.05$) whereas others are significant ($P < 0.05$). * $P < 0.05$, ** $P < 0.001$ a significant difference vs. normal group respectively. # $P < 0.05$, ## $P < 0.001$ represents a significant difference vs. HCC group respectively.

DISCUSSION

miRNA therapy is known for its high efficacy *in vitro*, but it requires more verification for implementation through *in vivo* studies. There is a shortage of reported data on miRNA-26a administration in laboratory animals. Therefore, the possible therapeutic benefit of miR-26a as a prophylactic agent against liver cancer formation was explored in our research. Herein, induction of HCC was associated with a marked increase in the concentration of both AFP and DCP (tumor markers) from weeks 8 to 20. HCC group that was treated with miRNA-26a significantly showed a drop in the concentration of tumor markers from weeks 8 to 20. These results are consistent with Yang and colleagues (2013), who have significantly demonstrated a reduction in the nodular tumor number and tumor weight in ovarian cancer mouse after the miRNA-506 administration. Furthermore, miRNA-16 injection with atelocollagen through the tail vein significantly prevented prostate tumor development in the bone (Takeshita *et al.*, 2010). In the current

HCC+miRNA-26a group, regression of hepatic malignant changes and recovery of a nearly ordinary lobular structure has been previously shown by kato *et al.* (2009), who found that delivery of miRNA-26a therapy significantly decreased the liver tumor burden of mice.

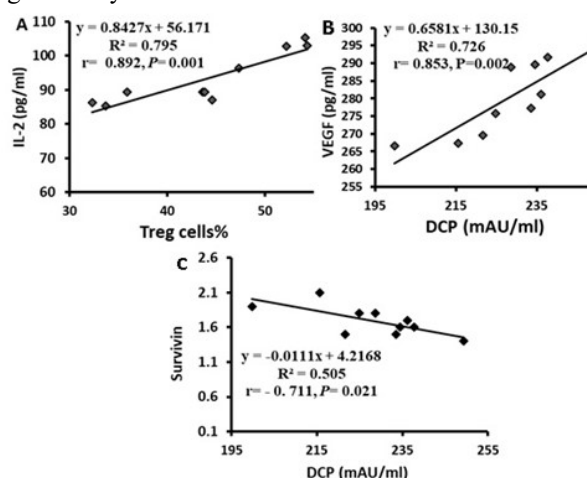


Fig. 5: The relationship between the various studied parameters in mice of HCC+miRNA-26a group at week 20. The frequency of Treg cells, serum levels IL-2 (pg/ml) (A) and VEGF (pg/ml) (B), the concentration of DCP (MAU/ml) (C) and relative expression of survivin. r : Pearson correlation coefficient. IL-2: Interleukin-2; DCP: Des-gamma-carboxy Prothrombin; VEGF: Vascular endothelial growth factor.

Tregs are of essential importance for immune tolerance, and higher Treg levels in peripheral blood can contribute to disease progression. In the present work, the elevated frequency of $CD4^+CD25^+$ Treg cells in HCC-induced mice is agreed with previous studies that demonstrated a relationship between the higher levels of Treg cells and a clinically higher number of tumors as well as higher levels of AFP (Sasaki *et al.*, 2008; Sun *et al.*, 2017). In murine HCC model, activated Treg cells demonstrated nine modified miRNAs and five miRNAs were significantly modified in the Treg cells that were transfected with siRNAs (Chen *et al.*, 2014). Signals that interfere with functional miRNAs of Treg cells can lead to autoimmune disorders (Zhou *et al.*, 2008). Administration of miRNA-26a, in our work, revealed a suppression impact on the frequency of Treg cells in the HCC+miRNA-26a group. Such findings indicate that miRNA-26a has been suggested to be a part of Treg cell activation and differentiation.

IL-2 is one of the primary cytokines that mainly secreted by $CD4^+$ Th cells as a classic-related Th1 cytokine but can also be produced in a lower extent by $CD8^+$ cells, natural killer cells, and activated dendritic cells (Liao *et al.*, 2013). In the current investigation, significant increased levels of IL-2 indicated the polarization towards Th1 immune responses along with HCC formation. In contrast, miRNA-26a administration caused a reduction of IL-2 levels and subsequently suppressed Th1

expansion. Accordingly, miRNA-26a can negatively control the secretion of miRNA-26a. Regarding the pleiotropic functions of IL-2, it is considered as a T cell growth factor, plays a key role in preservation of CD4⁺ Treg cells and participates in the differentiation of naïve CD4⁺ T cells into Th subsets (Jiang *et al.*, 2016). This activity of IL-2 was confirmed in the present work, whereas the HCC+miRNA-26a group showed a significant positive correlation between IL-2 levels and Treg cells during the 20th week ($r=0.892$, $P<0.001$).

Inflammation is a dynamic and important network of HCC changes and development and attraction site for other mediators (Liu *et al.*, 2018). The current work supports one of the dual roles of TNF- α as a tumor promoter, whereas the inflammatory TNF- α levels were significantly upregulated in HCC group. It was reported that TNF- α plays a crucial role in promoting HCC development (Schwabe and Brenner, 2006). The administration of miRNA26a in our HCC model may influence the immune reactions by reducing TNF- α serum levels, based on the proposed regulatory role of miRNA-26a.

IL-10 is a cytokine of phenotypic plasticity, can be secreted by Th2 cells, macrophages, and B-lymphocytes, with immunosuppressive actions that prevents macrophages from being activated and interferon (IFN)- γ secretion (Sabat *et al.*, 2010). The current results of elevated IL-10 levels are consistent with several reported studies that showed high levels of IL-10 in both the serum of HCC patients and tumor cells (Othman *et al.*, 2013), which suggest that IL-10 represents the liver inflammation degree and correlated with HCC development (El-Emshaty *et al.*, 2015). In this context, the synergy between the frequency of Treg cells and IL-10 levels in the current study during HCC propagation was also reported by HCC patients (Nishida and Kudo, 2017). In contrast to the expected decrease of IL-10 level after miRNA-26a administration, serum IL-10 levels in the HCC+miRNA-26a group recorded a pronounced elevation in comparison to the HCC group. The explanation of this result may be regarded to the immunomodulation effect of miRNA-26a via activating Th2 responses and eliminating Treg cells. Other studies supported this role of IL-10 by showing antitumor defense mechanisms that lead to stimulation of the cytotoxic T cells with IFN- γ and granzymes expression (Mumm *et al.*, 2011), as well as inhibition of IL-2 secretion by type 2 Th cells (Soltanzadeh-Yamchi *et al.*, 2018).

Survivin is one of the most effective protein inhibitors of apoptosis, implicated in the physiological and pathological consequences of various cancer cells (Jaiswal *et al.*, 2015). The rise in the expression of survivin level during the induction of HCC in the current research was consistent with the evidenced correlation between this anti-apoptotic marker and

hepatocarcinogenesis (Ding *et al.*, 2010), with a reduced survival rate of HCC patients (Idenoue *et al.*, 2005). MiRNA-26a downregulation in human MCF7 breast caused a decrease in apoptosis and favoring tumorigenesis (Zhang *et al.*, 2011). Levels of survivin were substantially decreased following miRNA-26a administration in our HCC-induced mice. This functional apoptotic activity of miRNA-26a delivery was also reported by Kato *et al.* (2009) who found direct inhibition of cyclin D2 and cyclin E2 expression in transfected HepG2 cells with miRNA-26a, and delivery of miR-26a by vector provoked specific apoptosis in tumor cells without a triggered death of ordinary hepatocytes via the *in vivo* model. Moreover, no detectable liver toxicity has been reported in normal animals that treated with vector delivery of miR-26a (Kato *et al.*, 2009). Moreover, the survival rate was improved in HCC patients with negative survivin expression (Ding *et al.*, 2010). Caspase-3 is one of the IL-1 β converter enzymes, a key factor involved in the regulation of apoptosis and inflammation of the cells, which can promote human tumorigenesis (Reed, 2000). Herein, it has been shown that miRNA-26a administration caused a downregulation of the higher expressed levels of caspase-3 compared to the HCC mouse model. This relation between liver tumor formation and active caspase-3 was linked to low survival rate in oral tongue squamous cell carcinoma (Liu *et al.*, 2017). Additionally, overexpression of caspase-3 was detected in four types of hepatoma cell lines and highly expressed in HCC tissue than normal liver tissue that was significantly correlated with AFP (Persad *et al.*, 2004). Thereby, the miRNA-26a defense effect prevented the development of tumor and controlled caspase activity in liver tissue of the present mouse model.

VEGF is a factor that plays a crucial role in angiogenesis, stimulating proliferation of endothelial cells and cancer cells expressing VEGF-receptor (Grothey and Galanis, 2009). The current HCC-induced model showed high serum levels of VEGF that has been previously recorded in HCC cell lines as and peripheral blood of HCC patients (Poon *et al.*, 2004). The regression of tumor growth that has been observed in the present miRNA-26a treated HCC-induced mouse was liked with significantly lower levels of VEGF compared to HCC group. The hindering effect of miRNA-26a on angiogenesis has been reported in previous studies whereas miRNA-26a deficiency in HepG2 cells resulted in fast motility and morphological changes like epithelial-mesenchymal transition, as well as pulmonary metastasis acceleration in xenograft nude mouse (Yang *et al.*, 2014; Ma *et al.*, 2016). miRNA-26a can reduce VEGFA via stimulating PIK3C2alpha/Akt/HIF-1alpha/VEGFA pathway (Chi *et al.*, 2013).

CONCLUSION

The present findings showed that miRNA-26a administration prevented liver tumor progression in the

HCC model. Furthermore, miRNA-26a may participate in the polarization and plasticity of CD4⁺T cells towards Th2-related immune responses and favoring adequate enhancement of anti-inflammatory responses through a substantial rise in IL-10 and the depletion of pro-inflammatory cytokines like TNF- α . It is preferentially down regulating the expression of pro-apoptotic markers and the reduction of angiogenesis. Therefore, the functional restoring of miRNA-26a has immunomodulatory, pro-apoptotic and angiogenic effects in the HCC mouse model. These collected data strongly indicate that implemented miRNA in a synthetic form is considered a promising targeted liver cancer therapy. Further studies are required to verify the clinical validity of miRNA-26a replacement.

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