Alterations of Some MicroRNAs Expression in Hepatocellular Carcinoma Cell Lines by Sodium Butyrate

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Abstract

Background: Epigenetic gene regulation is important in human cancer.

Aim of Study: We investigated the possible role of acetylation level in the regulation of microRNAs (miRNAs) expression in hepatocellular carcinoma.

Material and Methods: We first determined the inhibitory concentration (IC20, IC35 and IC50) of the histone deacetylase inhibitor Sodium Butyrate (SB) in HepG2 and Huh7 cells. Then, we analyzed the expression level of five miRNAs (miR-133b, miR-122-5p, miR-26a-5p, miR-539-5p and miR-518f-3p) after treatment using qRT-PCR assay. MiRTarBase, MiROB and GeneCards databases were used for the identification of strong validated targets of examined microRNAs, and the detection of possible functions of the selected targets and related pathways.

Results: SB revealed an anti-proliferative effect in both HepG2 and Huh7 cells, with IC50 values of 6.7mM and 9.2mM, respectively. MiR-133b showed up-regulation in HepG2 and miR-122-5p was down-regulated in HepG2 and Huh7 (29.5 and 1000 folds) in a dose dependent manner. MiR-26a-5p exhibited decrease in its level in both cell lines at IC35, but was increased at IC50 in HepG2 cells. MiR-518f-3p showed increase in its level during various doses of SB in both cell lines. Finally, miR-539-5p showed down expression in both cell lines. Prediction pathway analysis referred to the important role of each of miR-133b, miR-122-5p and miR-26a-5p in proliferation, apoptosis, angiogenesis and metastasis.

Conclusion: Sub-lethal doses of SB have significant effects on miRNAs expression in human hepatocellular carcinoma cells. Understanding the epigenetic regulation of miRNAs may enhance the development of therapeutic strategies against HCC.

Key Words: HCC – microRNAs – Epigenetics – Acetylation – Sodium butyrate.

Introduction

HEPATOCELLULAR Carcinoma (HCC) is the fifth most prevalent cancer disease worldwide [1].

Chronic liver injury, that results in inflammation, hepatocyte regeneration, liver matrix transforming, fibrosis, and, finally, cirrhosis can cause HCC development [1]. In addition to numerous genetic causes, the development and progression of HCC is strongly correlated with epigenetic pattern of DNA and histone modifications. In the last decade, Histone Acetylases (HATs) and Histone Deacetylases (HDACs) have shown important roles in the regulation of transcription [3]. Histone Deacetylase Inhibitors (HDACi) can affect angiogenesis, cell differentiation and apoptosis [4].

Sodium Butyrate (SB) is one of the short chain fatty acids naturally formed in gastrointestinal tracts. SB can cause cell cycle arrest, apoptosis and stimulate cell differentiation in multi type of cancer cells lines notably the colon [5], breast [6], liver [7]. SB, a histone deacetylases inhibitor, can induce broad alteration in chromatin structure and expression of genes [11]. SB has been shown to inhibit proliferation in different cancer cell lines high efficiency and very low general toxicity [8-11].

It has been shown that histone deacetylation plays a vital role as a regulator for microRNAs (miRNAs) expression [12]. MiRNAs are a small non-coding RNAs of ~22 nucleotides that regulate gene expression machinery. There is rising attention in recognizing the harmony between miRNA expression in cancer disease, chemo-and radio-sensitivity to enhance the performance of the treatment manner through prediction or modulation of sensitivity or response [13].

The goal of the present study is to investigate the effects of acetylation level alterations by SB on HCC cell lines survival and incontrolling the expression of five miRNAs, known to be involved
incell proliferation, apoptosis, angiogenesis and metastasis.

Material and Methods

Cell lines:
The human HCC cell lines, HepG2 and Huh7 used in the current study, were obtained from VAC-SERA (the Egyptian Company for Production of Vaccines, Sera and Drugs). The cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA), 100U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator (Thermo Scientific, City, USA). SB was obtained from Sigma-Aldrich (Merck KGaA, Germany). SB stock solution was prepared at a concentration of 250mM in distilled water and stored at ~20°C.

This study was conducted at National Cancer Institute, Cairo University (NCICU) from August 2015 – December 2017.

Cell viability assay:
The cytotoxic effect of SB was determined using SulphoRhodamine-B assay (SRB) as described previously [14]. Cells were seeded onto 96-well plates (5 X 10³/well) corresponding to 75% confluence, for 24h. The cells were treated with 7 different concentrations of SB (0.25, 0.5, 2, 5, 6, 8 and 12mM) and incubated at 37°C in a humidified 5% CO₂ incubator for another 48h. Untreated cells (control cells) were seeded in parallel in serum free media. After incubation, cell fixation was done by treatment with TCA to a final concentration 10% for 30min. SRB (0.4%) was added for 30min, then cells were washed with 1% Acetic acid. 10mM Tris base were added and the absorbance was recorded on a microplate reader (Tecan Sunrise, Austria) at a wavelength of 570nm. Each treatment was done in triplicates. The sub-lethal inhibitory concentrations of SB (IC20, IC35 and IC50) were calculated and selected for use in the following experiments as shown in (Table 1) and Fig. (1).

MiRNA extraction and quantitative real-time PCR analysis:
Total RNA (including miRNAs) from HepG2 and Huh7 cell lines, before and after treatment with SB, was extracted using miRNeasy kit as described in the manufacturer's instructions (Qiagen, Germany). RNA was purified using miRNeasy MinElute spin columns (Qiagen, City, Country) then eluted in 35 µl RNAse-free water. RNA concentrations were quantified using NanoDrop-Onespectrophotometer (Thermo Scientific, City, USA). Total RNA (250ng/sample) was reverse-transcribed using miScript RT kit (Qiagen). Reactions were incubated at 37°C for 1h followed by inactivation of the reaction by incubation at 95°C for 5min.

The expression levels of miR-133b, miR-26a-5p, miR-122-5p, miR-518f-3p and miR-539-5p were measured using miScript SYBER Green reagent kit (Qiagen) according to the manufacturer’s protocol. About 5ng of cDNA was used as template in a 10 µl PCR reaction containing 1X SYBR Green master mix, 1XmiRNA specific forward primer, and 1X universal primer. The conditions for qRT-PCR were as follows: 95°C for 10min, followed by 40 cycles of 95°C for 15s and 55°C for 1min. All the RT-qPCR reactions were performed on ViiA7 real-time PCR system (Applied Biosystems, USA). All samples were performed in duplicate. U6 snRNA was used as an endogenous control for normalization. The relative expression analysis of miRNAs studied was performed using comparative Ct method [15].

Data analysis:
The obtained real time data was analysed using ΔΔCt comparative method. U6 was used as an endogenous control and its Ct values were subtracted from the examined miRNA Ct values to obtain ΔCt. The fold of change was calculated from the following formula: Fold of change = 2^-ΔΔCt.

Statistical analysis:
The significant difference in cytotoxicity assay values was evaluated using one-way ANOVA, followed by the Dunnett's multiple comparison test. Data are presented as means ± SD. Statistical analyses were performed using GraphPad Prism Version 5 for windows (GraphPad Software Inc., city, USA). p-value <0.05 was considered significant.

Bioinformatics analysis:
MiRTarBase database (http://mirtarbase.mbc.nctu.edu.tw/php/index.php) (January 2018) and MirOB database (http://mirob.interactome.ru/) (January 2018) were used to identify the experimentally validated targets and the related pathways of the examined miRNAs in this study. GeneCards database (http://www.genecards.org/) has been adopted to identify possible functions of the validated targets.

Results
In this study we investigated the cellular effects of three sub-lethaldoses of SB treatment on liver
cancer cell lines (HepG2 and Huh7) survival and on the expression level of 5-selected miRNAs.

**Cytotoxicity of SB on liver cancer cell lines (HepG2 and Huh7):**

To determine the effect of SB on HepG2 and Huh7, cells were exposed to 7 concentrations of SB ranging from (0.25, 0.5, 2, 5, 6, 8, and 12mM) for 48h, and then cell viability were measured using SRB assay. Our data revealed a concentration-dependent decrease in the survival rate with SB treatment Fig. (1) in both cell lines examined. Notably, HepG2 cells exhibited a higher sensitivity to SB treatment compared to Huh7. Detected IC20, IC35, and IC50 concentrations were used for further analyses Fig. (1) and (Table 1).

**Quantitative RT-PCR assay:**

The effect of three different concentrations of SB (IC20, IC35, and IC50) on the expression level of selected miRNAs was examined. The examined miRNAs revealed different expression level between the two cell lines (Table 2).

**Pathway predication:**

miRTarBase, MirOB and Gene Cards have (Janaury 2018) been searched to identify experimentally validated targets of selected miRNAs and their suggested functions and related pathways. Interestingly, the revealed target genes signaling are involved, to some extent, in cell death, proliferation and survival.

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Target gene</th>
<th>Gene function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hsa-miR-133b</td>
<td>BCL2L2</td>
<td>• Involved in intrinsic anti-apoptotic pathway.</td>
<td>[16,17]</td>
</tr>
<tr>
<td></td>
<td>MCL1</td>
<td>• Acts as anti-apoptotic.</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>IGF1R</td>
<td>• Acts as anti-apoptotic.</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>FAIM</td>
<td>• Involved in extrinsic anti-apoptotic pathway.</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>FSCN1</td>
<td>• Involved in metastasis.</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>EGFR</td>
<td>• Induces proliferation.</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td>FGFR1</td>
<td>• Involved in migration and angiogenesis.</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>MET</td>
<td>• Acts as oncogenic and involved in resistance to drug.</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>GLI1</td>
<td>• Induces migration.</td>
<td>[24]</td>
</tr>
<tr>
<td>Hsa-miR-26a-5p</td>
<td>HMGA1</td>
<td>• Involved in metastasis.</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>MTDH</td>
<td>• Involved in tumor cell expansion and metastasis.</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>FGFR9</td>
<td>• Induces cell survival, tumor growth and invasion.</td>
<td>[27]</td>
</tr>
<tr>
<td></td>
<td>HGF</td>
<td>• Involved in angiogenesis, tumorigenesis and tumor regeneration.</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>PIK3CA2A</td>
<td>• Induces proliferation and cell survival.</td>
<td>[29]</td>
</tr>
<tr>
<td>Hsa-miR-122-5p</td>
<td>IGF1R</td>
<td>• Involved in cell growth and survival control, induces tumor transformation</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>and survival of malignant cell.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MAPK11</td>
<td>• Involved in cellular proliferation.</td>
<td>[31]</td>
</tr>
<tr>
<td></td>
<td>AKT3</td>
<td>• Regulates many processes including metabolism, proliferation, cell survival,</td>
<td>[32]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>growth and angiogenesis.</td>
<td></td>
</tr>
<tr>
<td>Hsa- miR-539-5p</td>
<td>BCL2L2</td>
<td>• Involved in intrinsic anti-apoptotic pathway.</td>
<td>[33]</td>
</tr>
<tr>
<td></td>
<td>TWIST1</td>
<td>• Promotes tumor cell invasion and metastasis.</td>
<td>[34]</td>
</tr>
<tr>
<td></td>
<td>ZEB1</td>
<td>• Induces an Epithelial-Mesenchymal Transition (EMT) and promotes tumorigenicity by repressing stemness-inhibiting microRNAs.</td>
<td>[35]</td>
</tr>
<tr>
<td>Hsa-miR-518f-3p</td>
<td>RBM8A</td>
<td>• Inhibits formation of proapoptotic isoforms such as Bcl-X.</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>STK4</td>
<td>• Act as pro-apoptotic kinase.</td>
<td>[36]</td>
</tr>
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<td></td>
<td>TYRO3</td>
<td>• Has role in controlling cell survival and proliferation, spermatogenesis,</td>
<td>[36]</td>
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<td></td>
<td></td>
<td>immunoregulation and phagocytosis.</td>
<td></td>
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<tr>
<td></td>
<td>RPS6KA3</td>
<td>• Regulates diverse cellular processes such as cellular growth, motility,</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>survival and proliferation.</td>
<td></td>
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</table>
Figure (1): Viability of HepG2 and Huh7 cells at different doses of SB treatment for 48h. The results are expressed as the mean ± SD of 3 separate experiments.

Figure (2): Cellular viability of HepG2 and Huh7 cells treated with different doses of SB for 48h. (A) Control untreated cells, (B) Cells treated with IC20 of SB, (C) Cells treated with IC35 SB, and (D) Cells treated with IC50 of SB.

Discussion

The histone deacetylase inhibitor (HDACi), Sodium Butyrate, is a component of short-chain fatty acids produced by anaerobic fermentation of dietary fibers in the gastrointestinal tract of mammalian species [37]. SB can inhibit cell proliferation and potentiate cancer therapy and prevention via inhibition of HDACs, that makes it a good candidate for cancer management. It has been shown that HDACi play a vital role as regulators for many miRNAs [38]. Thus, understanding their specific role could changes the therapeutic strategies for cancer.

The present data displayed decreases in cell growth of HepG2 and Huh7 liver cell lines when treated with SB in a dose-dependent manner, which is consistence with previous studies [9]. Furthermore, HepG2 cells revealed higher sensitivity upon treatment with SB than Huh7 cells, their IC50 values were 6.7mM and 9.2mM, respectively. Previous studies have reported that cytotoxicity of SB was in the range of 1.47mM to 5mM for HepG2 [11,39] and 2mM in Huh7 [40] which are different from our detected IC50 values. This might be due to the difference in the number of passages of each cell line. Meanwhile, the variation between the two cell lines might be attributed to the variation in their biological and genetic characteristics such as difference in p53 expression as well as expression of certain proteins such as α-feto protein, and others in HepG2 cells [41].

It has been shown that SB might induce profound changes in miRNA expression of HCC cells [42]. MiRNA scan have extensive effects through regulation of a variety of genes that are involved...
in the most vital cellular processes including, differentiation and cell cycle regulation [43,44].

The present data shows an up-regulation of miR-133b expression level in HepG2 cells, treated with SB, in a dose-dependent manner whereas, it is down-regulated in Huh7 with low dose treatment. Previous studies have shown that treatment of gastric cancer cell lines with SAHA (HDAC inhibitor) increases the expression level of miR-133b/a-3p and decreases acetylation which refers to the role of histone acetylation in regulation of miR-133b expression [21]. Our gene target analysis by GeneCards database suggests that miR-133b might have a pro-apoptotic function as it seems to target a number of anti-apoptotic genes (Table 3). This is consistent with several studies that have shown down regulation of miR-133b level in different types of cancer [45,46] and this change was significantly associated with poor survival and distant metastasis in colorectal carcinoma [47]. Overexpression of miR-133b can boost apoptosis through TRAIL pathway as detected in HeLa cells [19]. Apoptosis can be also induced via controlling the expression of Bcl2 family as shown in lung cancer and bladder cancer by mir-133b [48]. MiR-133b can affect cell proliferation and apoptosis through suppressing c-Met signaling pathways in colorectal carcinoma [49]. Previous study showed that miR-133b can inhibit target gene silent information regulator 1 (Sirt1) that lead to suppression of cell proliferation and invasion in hepatocellular carcinoma [50]. Down-regulation of miR-133b was significantly correlated with primary resistance ovarian carcinomas [51] which refers to its crucial role in chemotherapeutic efficacy of cancer. On the other hand, a recent study showed that miR-133b can reduce the phosphorylation of Erk1/2 and Akt by targeting EGFR in ovarian cancer [21].

The role of miR-26a-5p in carcinogenesis appears to be more complicated as it has both oncogenic and tumor suppressive effect in cancers including hepatocellular carcinoma cells [39]. Using HDAC inhibitor, up-regulation of miR-26a was recorded in human umbilical cord-blood multipotent stem cells (hUCB-MSCs) that plays important roles in cellular senescence via targeting high-mobility group A2 protein (HMGA2) [52]. It was shown that miR-26a acts as tumor suppressor in a variety of cancers [53,54]. MiR-26a-5p is often downregulated in several tumor tissues and tumor cell lines [54]. MiR-26a was able to suppress the proliferation, migration, and invasion of cells via negative regulation of MTDH, which supported the role of miR-26a as tumor suppressor in HCC cells [55]. In addition, it was found that miR-26a has the potency to suppress tumor growth and metastasis by regulating FGF9 [56]. On the other hand, miR-26a was found to enhance metastasis of lung cancer by directly targeting the tumor suppressor PTEN and thus activating AKT signaling pathway [26].

Our results showed a down regulation of miR-26a at low doses treatment (IC20 and IC35) whereas it was up regulated at high doses (IC50) in hepatocellular cells which is correlated with inhibition of cell proliferation. This might be due to the dual effect of SB, which means low concentrations of SB can produce a significant increase in cell growth in cell line, while unlikeness high concentrations of it can inhibit cell growth in the same cell line [57].

MiR-122-5p has been found to be closely associated with HCC [58]. In this study, we found that miR-122-5p was significantly down regulated in HepG2 and Huh7 cells treated with different concentrations of SB compared to control cells. Down expression of miR-122-5p was reported to cause inhibition of HCC cell proliferation, migration, and invasion by the targeting of IGF1R, MAPK11 and BCL2L2. BCL2L2 is one of the main inhibitors to intrinsic apoptotic pathway by reducing the activation of some essential pathways, such as c-Myc, IGF, and Wnt/β-catenin [59]. MiR-122-5p inhibits Akt3, which regulates many processes including metabolism, proliferation, cell survival, growth and angiogenesis [60]. The metastatic and invasive property of HCC cells was also demonstrated to be suppressed by miR-122 in an ADAM17-dependent mechanism [31]. In addition, other studies revealed that miR-122 can induce apoptosis by inhibiting Bcl-w transcription [61].

Although miR-539-5p has been shown to be involved in other types of cancers, our study showed significant downregulation in both cells treated with different concentrations of SB compared with untreated cells. Other studies reported that miR-539-5p was downregulated in osteosarcoma and suppresses tumor metastasis by targeting MMP8 and acts as an oncogene or tumor suppressor in different types of cancer [62].

MiR-518f-3p was significantly up regulated in HepG2 and Huh7 cells treated with different concentrations of SB compared with untreated cells, which is in agreement with one study that showed a significant up-regulation of miR-518f-3p in HCC [63]. According to miRTarebase and Gene Cards database miT-518f-3p inhibited TYRO3 and RPS6KA3. Other studies showed that TYRO3
is controlling cell survival and proliferation in breast cancer [64]. Further studies are required to understand the possible role of miR-518f-3p in HCC.

Taken together, our results suggested that the biological effects at different concentrations of SB might have different mechanisms at the cellular machinery level. Further studies are needed to elucidate this observation and its potent therapeutic effects for HCC.

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