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Research Paper

Butyrate induces ROS-mediated apoptosis by modulating miR-22/SIRT-1 pathway in hepatic cancer cells

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ABSTRACT

Butyrate is one of the short chain fatty acids, produced by the gut microbiota during anaerobic fermentation of dietary fibres. It has been shown that it can inhibit tumor progression via suppressing histone deacetylase and can induce apoptosis in cancer cells. However, the comprehensive pathway by which butyrate mediates apoptosis and growth arrest in cancer cells still remains unclear. In this study, the role of miR-22 in butyrate-mediated ROS release and induction of apoptosis was determined in hepatic cells. Intracellular expression of miR-22 was increased when the Huh 7 cells were incubated with sodium butyrate. Over-expression of miR-22 or addition of sodium butyrate inhibited SIRT-1 expression and enhanced the ROS production. Incubation of cells with anti-miR-22 reversed the effects of butyrate. Butyrate induced apoptosis via ROS production, cytochrome c release and activation of caspase-3, whereas addition of N-acetyl cysteine or anti-miR-22 reversed these butyrate-induced effects. Furthermore, sodium butyrate inhibited cell growth and proliferation, whereas anti-miR-22 inhibited these butyrate-mediated changes. The expression of PTEN and gsk-3 was found to be increased while p-akt and β-catenin expression was decreased significantly by butyrate. These data showed that butyrate modulated both apoptosis and proliferation via miR-22 expression in hepatic cells.

1. Introduction

The short chain fatty acids, namely butyrate, acetate and propionate, produced by the gut microbiota during anaerobic fermentation are mainly absorbed by the colon or liver cells [1]. Butyrate exerts several effects including, induction of apoptosis and growth arrest in patients with solid tumors [2,3]. It delays tumor growth and induces apoptosis by mediating expression of histone deacetylase (HDAC), SIRT-1, caspase 3, and NFκB [4]. Furthermore, several HDAC inhibitors including butyrate have been found to be useful in chemotherapy strategy to restrain proliferation, growth, and to provoke cell death in the cancer cells [3,5]. Previously it was shown that butyrate significantly inhibited CD44 expression, thereby inhibiting the metastatic ability of the human colon carcinoma cells [6]. Prolonged butyrate treatment inhibited the pro-MMP-2 activation and tumor cell migration potential of HT 1080 tumor cells [7]. Although several scanty reports show the mechanisms by which butyrate mediates apoptosis in cancer cells, no clear relationship is available between butyrate and the induction of apoptosis.

SIRT-1, a member of histone deacetylase protein family, is widely expressed in the hepatoma cells which helps in the tumor cell survival and growth [8]. SIRT-1 knockdown enhanced the chemosensitivity of H292 cells to cisplatin and reduced the tumor volume and the metastatic ability of the cells in nude mice [9]. Recently it was shown that SIRT-1 expression was associated with nanog expression in patients with colorectal adenocarcinoma [10]. Several anticancer drugs and chemotherapeutic agents target the expression of SIRT-1 to suppress the tumor growth [11]. Butyrate, being a HDAC inhibitor, is not known whether it regulates apoptosis via SIRT-1 in hepatic cells.

miRNAs are small, ~21–23 nucleotides long, non-coding RNAs that inhibit the target mRNAs at post-transcriptional level. Depending on the degree of complementarity, miRNA binding with target mRNAs induces either mRNA degradation or inhibits translation, thereby regulating a variety of cell functions, such as proliferation, apoptosis, senescence, differentiation, and cancer [12]. Several specific miRNAs have been shown to act as tumor suppressors or as oncogenes in

List of abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); DiOC6, 3,3′-dihexyloxacarbocyanine iodide; DMSO, Dimethyl sulfoxide; ECL, Enhanced chemiluminescence; Gpx, Glutathione peroxidase; H_2-DCFHDA, 2′,7′-dichlorodihydrofluorescein diacetate; HDAC, Histone deacetylase; NAC, N-acetyl cysteine; ROS, Reactive oxygen species; SOD, superoxide dismutase; TUNEL, Terminal deoxynucleotide transferase dUTP Nick End Labeling

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cancers [13]. Recently, miRNA-204-5p was shown to regulate hepatocellular progression via down-regulating SIRT-1 [14]. miRNA-34a inhibited cell proliferation in prostate cancer by downregulating SIRT-1 expression [15]. In glioblastoma cells, miR-22 inhibited the proliferation, motility and invasion by targeting SIRT-1 [16]. Although, butyrate had been shown to inhibit the cell proliferation and tumor growth, there is no data available to show the involvement of miRNA in butyrate-mediated apoptosis. In the present study, we demonstrated that butyrate induces apoptosis through up-regulation of miR-22, followed by the downregulation of SIRT-1, resulting in increased ROS production and apoptosis in hepatic cells.

2. Materials and methods

2.1. Cell culture, butyrate treatment and miRNA transfection

Huh 7 cells (1×10^5 cells/well) were cultured in 6-well plates containing DMEM medium with 10% FBS and 1% penicillin-streptomycin for 24 h. The cells were incubated with varying concentrations (0–8 mM) of sodium butyrate for 24 h under serum-free conditions. Transfection of miR-22 premiRs, anti-miR-22 oligos or non-specific miRNA (NS) (Sigma-Aldrich, St Louis, MO, USA) was performed using siPORT™ NeoFX™ transfection reagent (Thermofisher, Carlsbad, CA, USA) as described by us previously [17]. After the experiments, the cells were collected for either total RNA isolation or protein separation.

2.2. Western blotting analysis

Whole cell lysates prepared in M-PER mammalian protein extraction reagent (Thermofisher) and complete protease inhibitor cocktail (1:100). Equal amount (30–50 μg/well) of protein samples were separated on SDS-PAGE and transferred to PVDF membranes. The membranes first blocked with 10% non-fat dry milk and incubated with the primary antibodies (SIRT-1, PTEN, akt, p-akt, gsk3β, β-catenin, bcl-2, cytochrome c, caspase-9, caspase-3 and β-actin). After incubation with appropriate secondary antibodies, the immunoblots were incubated with ECL plus Western blotting substrate (Thermofisher), exposed to X-ray films and developed. PVDF membranes were reused after removing the antibodies using Restore plus Western blot stripping buffer (Thermofisher).

2.3. miRNA isolation and real-time RT PCR

Total RNA enriched with miRNA was isolated using miRvana RNA isolation kit (Thermofisher), cDNA was synthesized using cDNA synthesis Kit (Exiqon, Vedbaek, Denmark) and real time RT-PCR was performed using SYBRgreen mastermix and respective miRNA primers. miRNA-103a was used as a control RNA in all real time RT-PCR experiments. Intracellular expression of superoxide dismutase 1 and β-actin (as control) was quantitated by real time RT-PCR. The fold change in miRNA expression was determined by the comparative CT method ($2^{-\Delta\Delta CT}$) [18].

2.4. Colony formation assay

A total of 1000 cells per well were plated into 6-well cell culture plates and incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 3–5 days and the colony formation was observed. The colonies were stained with 0.1% crystal violet (Sigma Aldrich; dissolved in 50% methanol and 10% glacial acetic acid) and the colony formation was observed under the inverted phase contrast microscope.

2.5. Cell proliferation assay

Cells were seeded at ~2000 cells/well in 96-well plates. After 24 h, the cells were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. Cell viability was determined using the CellTiter-Blue cell viability assay kit (Promega Corp., Madison, WI, USA) as per the manufacturer's instructions. The luminescence was measured using microplate reader (BioTEK, Vinooski, VT, USA) and the percentage of proliferation was calculated by comparing with the control cells.

2.6. ROS analysis

Huh 7 cells (2×10^5 cells/well) were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. H_2-DCFDA (10 μM; Thermofisher) was added to each well and incubated for a further period of 60 min. The cells were observed under fluorescence microscope and the images were acquired. After incubation, cells were counterstained with DAPI and the images were taken using the fluorescence microscope. The percent of ROS production was calculated by counting in at least 10 different high power fields, averaged and the results were presented.

2.7. Measurement of mitochondrial membrane potential

The cells (4×10^5 cells/well) were incubated with butyrate alone or miR-22 alone or butyrate and anti-miR-22 together for 24 h. The cells were fixed by 4% paraformaldehyde, washed and 100 nM $DiOC_6$ was incubated for 15 min in dark as described previously [19]. The cells were washed using phosphate buffered saline and observed under fluorescence microscope.

2.8. ABTS assay

In order to quantitate the total free radicals scavenging activity or the total cellular antioxidants, the cells (2×10^5 cells/well) were plated and incubated with butyrate alone butyrate and anti-miR-22 oligos for 24 h. The cell lysates were prepared and equal amount of protein was incubated with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS, 200 μl of 7 mM) for 5 min at room temperature [20]. The absorbance was measured at 730 nm using water as blank. The percentage of ABTS scavenging activity was determined by the following formula.

$$\%\text{ABTS inhibition} = 1-\frac{Absorbance\ of\ the\ Sample}{Absorbance\ of\ Blank}X100$$

2.9. SOD assay

Huh 7 cells (2×10^5 cells/well) were plated and incubated with butyrate alone butyrate and anti-miR-22 oligos for 24 h. SOD activity was measured in the cell lysates using SOD assay kit (Sigma Aldrich) as per the manufacturer's protocol. The percentage of SOD activity was calculated.

2.10. TUNEL (Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay

Huh 7 cells (2×10^5 cells/well) were incubated with butyrate alone or butyrate and N-acetyl cysteine or butyrate and anti-miR-22 oligos for 24 h. After incubation the cells were washed, fixed using 4% paraformaldehyde and the apoptosis was detected using the *In Situ* Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) as per the manufacturer's instructions. The cells were mounted using Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and the cells were counted in at least 10 different high power fields and the percent of apoptosis induction was calculated.

2.11. Annexin V-PI assay

Huh 7 cells (2×10^5 cells/well) were incubated with butyrate alone or butyrate and N-acetyl cysteine or butyrate and anti-miR-22 oligos for 24 h. After incubation the cells were detached using 0.05% trypsin from the plates, washed and incubated with Annexin V-conjugated with fluorescein isothiocyanate (FITC) and propidium iodide for 30 min. Apoptosis positive cells were counted using flow cytometry (BD Biosciences, USA).

2.12. Statistical analysis

All the experiments were conducted in duplicates and 3 times and Graph Pad Prism 5.0 software was used. Comparisons among multiple groups were done using one-way ANOVA with and the Student's *t*-test was done to compare between two groups. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Butyrate induces the miR-22 expression

Being the histone deactylase inhibitor and regulator of many epigenetic factors, butyrate inhibits the cancer proliferation. Therefore, first the regulation of butyrate, acetate and propionate on the key miRNAs regulated by short chain fatty acids was analyzed. Fig. 1**A** shows that heat map generated by DataAssist™ software (Thermofisher) using the Ct (cycle threshold) values generated from the real time RT-PCR. The results showed that there was a differential regulation of miRNAs by short chain fatty acids. Fig. 1**B** shows the expression profile of the different miRNAs (22, 451, 30a, 21, 194, 26a and 150) in the presence of butyrate (5 mM). There was a significant increase in the intracellular expression of miR-22 (8.2 fold) by butyrate incubation. Hence, the role of butyrate on apoptosis and the role of miR-22 was in this pathway was studied. Furthermore, it was observed that the intracellular expression of miR-22 by butyrate (1–5 mM) was concentration dependent (Fig. 1**C**).

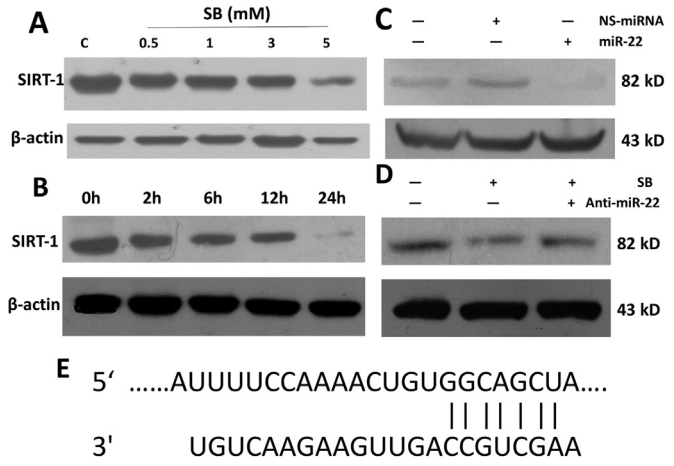

Fig. 2. Butyrate inhibits SIRT-1 expression via miR-22. Western blots were performed for SIRT-1 expression and β-actin was used as an internal control in all the experiments. The pictures are representative from 3 experiments. **(A)** The cells were incubated with different concentrations of butyrate (0.5–5 mM). **(B)** The expression pattern of SIRT-1 at different time periods (0–24 h) with butyrate (5 mM). **(C)** Effect of miR-22 on SIRT-1 expression was shown (NS-miR, non-specific miRNA). **(D)** Effect of anti-miR-22 on SB-induced SIRT-1 expression. **(E)** The target sequence of the miR-22 was positioned at 530–537 of 3′ UTR region of SIRT-1.

3.2. Butyrate Inhibits SIRT-1 expression via miR-22

Incubation of Huh 7 cells with butyrate inhibited the SIRT-1 expression in a concentration-dependent manner (1–5 mM) and inhibition at 5 mM concentration was most significant (73% inhibition) (Fig. 2**A**). Sodium butyrate (5 mM) was incubated with cells for different time periods for up to 24 h and the data showed maximum inhibition of SIRT-1 was found at 24 h (90% inhibition) (Fig. 2**B**). Next, the effect of miR-22 on SIRT-1 expression was determined. The data indicated that there was a down-regulation of SIRT-1 expression (Fig. 2**C**). Previously it was reported that miR-22 inhibited the SIRT-1 expression [21] and butyrate had also been shown to inhibit the SIRT-1

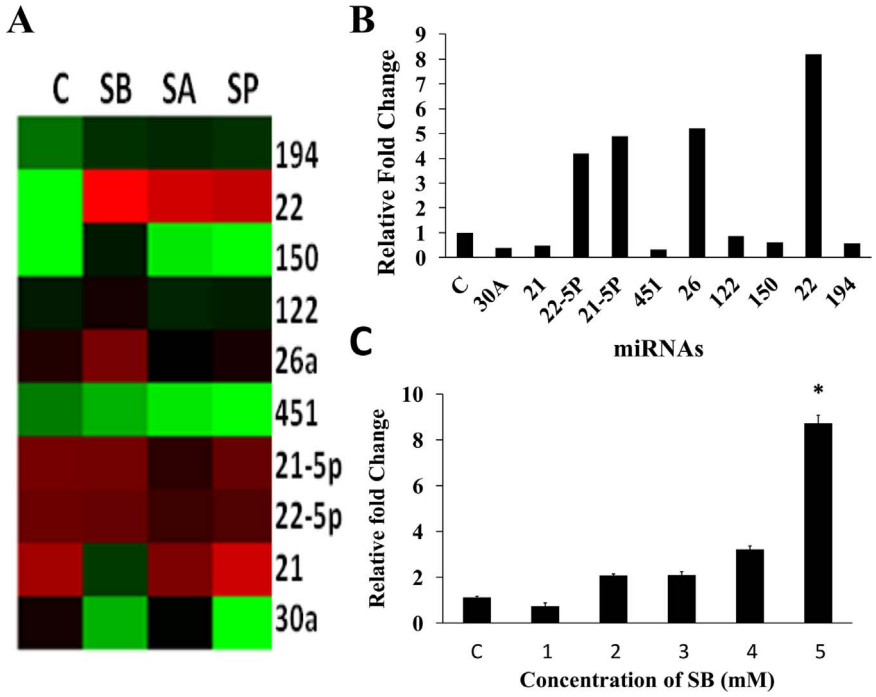

Fig. 1. Butyrate induces miR-22 expression. **(A)** The Heat map generated from the real time RT-PCR analysis using CT values from the expression profile of different miRNAs upon treatment with short chain fatty acids (5 mM) (SA, sodium acetate; SP, sodium propionate; and SB, Sodium butyrate). **(B)** The expression profile of the various miRNAs with the treatment of SB. (n=3) **(C)** The treatment of cells with different concentrations of SB (1–5 mM) on the expression of miR-22. (n=3; $p < 0.01$).

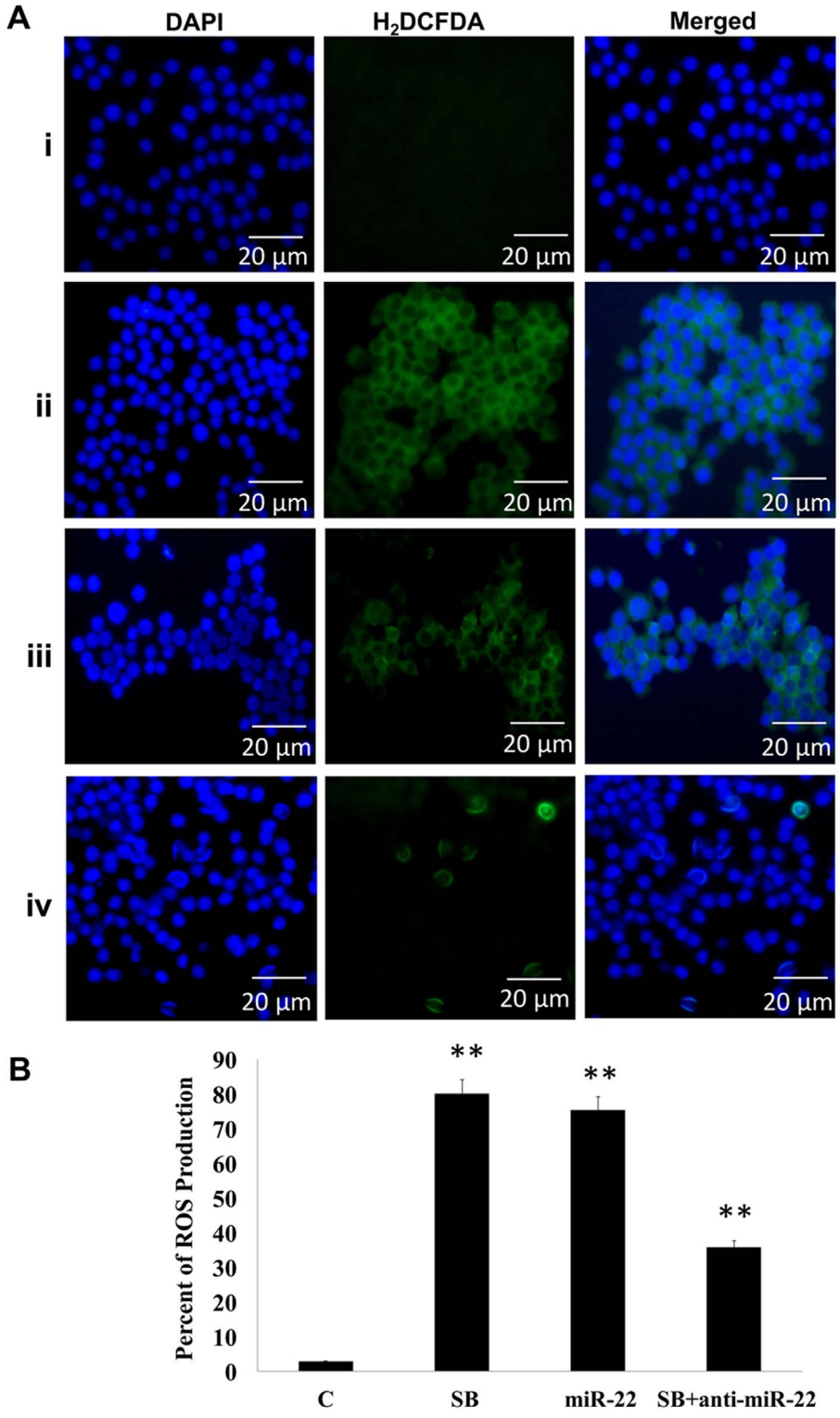

Fig. 3. A. Butyrate induces ROS production. Huh 7 cells were treated with butyrate alone or miR-22 alone or butyrate and ani-miR22 together. i, control; ii, Butyrate (5 mM); iii, miR-22; and iv, SB + anti-miR-22. **B.** The positive cells and the total cells were counted in at least 10 different high power fields and the average is calculated and presented as percent of ROS production. (n=3; $p < 0.01$).

expression [22]. Therefore, we hypothesized that the increased intracellular expression of miR-22 might be responsible for the decreased expression of SIRT-1. While butyrate inhibited (93% decrease) SIRT-1 expression, co-incubation of anti-miR-22 with butyrate reversed SIRT-1 expression (Fig. 2**D**). The miR-22 was found to be a direct target for SIRT-1 gene at 3′ UTR (positioned at 530–537) (Fig. 2**E**).

3.3. Butyrate induces ROS production

SIRT-1 was shown to inhibit the ROS generation [23] via regulating the expression of the cellular antioxidant genes. Hence, the effect of SIRT-1 inhibition on ROS release was tested using H_2DCFDA and the results showed that both butyrate and miR-22 induced ROS release,

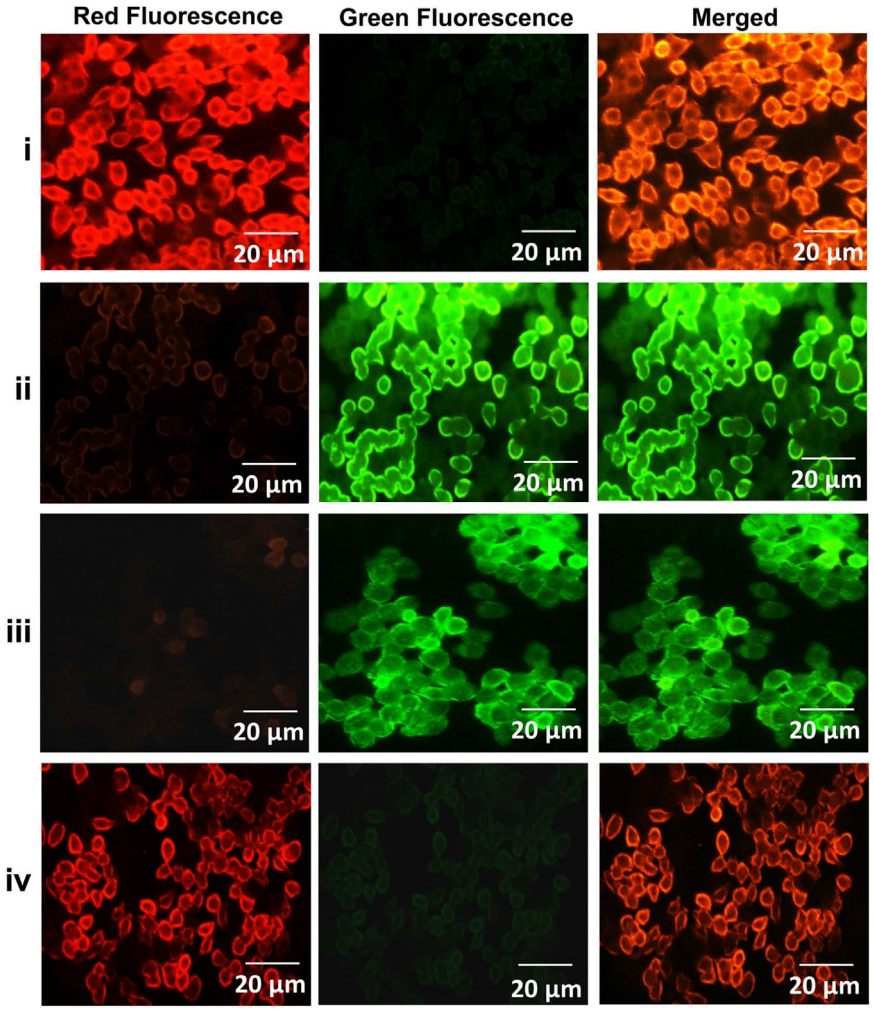

Fig. 4. Butyrate alters mitochondrial membrane potential (ψ_m). The cells were incubated with butyrate alone or miR-22 alone or anti-miR-22 with butyrate for 24 h and stained with DiOC6 dye for 5 min. The colour change to green colour was quantified using fluorescence microscopy. The left panel shows the pictures in green filter and the middle panel shows cells in red filter and the right panel shows the merged pictures. i, control; ii, Butyrate (5 mM); iii, miR-22; and iv, SB+anti-miR-22. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

while co-incubating butyrate with anti-miR-22 reversed ROS generation (Fig. 3**A**). The results showed that 5 mM butyrate or miR-22 incubation induced the cellular ROS level by 80.1% and 70.2% respectively. However, the intracellular ROS production was significantly inhibited when butyrate was co-incubated with anti-miR-22 (Fig. 3**B**).

3.4. Butyrate alters the mitochondrial membrane potential

Next, whether butyrate alters the mitochondrial membrane potential was determined. After 5 min of DiOC6 addition, both the red colour and green colour were observed under fluorescence microscope. In normal cells, DiOC6 resides in the cytoplasm and appears as red, and if there is any change in mitochondrial membrane potential (Ψ_m), the dye moves inside the mitochondria and appears green. The results showed that incubation of cells with either butyrate alone or miR-22 alone showed increased green colour compared to control, while addition of anti-miR-22 with butyrate did not result significant change in Ψ_m compared to control (Fig. 4**).**

3.5. Butyrate induces ROS-mediated intrinsic apoptosis

ROS is known to induce apoptosis via mitochondrial pathway in various cancer cells. To test whether butyrate induces apoptosis via mitochondrial pathway, the cells were incubated with butyrate alone, miR-22 alone or co-incubated with butyrate and anti-miR-22 and the signalling pathway involved in apoptosis induction was elucidated. Transfection of miR-22 premiRs decreased the expression of bcl2 (0.13 fold), while it increased the expression of cytochrome c (6.13 fold), cleaved-caspase 9 (14 fold) and cleaved caspase 3 (18.3 fold). Addition of NAC in miR-22 transfected cells resulted in increased bcl2 expression (2.8 fold) and decreased expression of cytochrome c, cleaved caspase-3 and cleaved caspase-9 (3.37, 4.7 and 6.1 fold respectively) compared to miR-22 transfected cells (Fig. 5**A**).

To study whether the butyrate-mediated effects are through miR-22, the cells were transfected with anti-miR-22 oligos followed by butyrate incubation. The results showed that butyrate inhibited the expression of bcl-2 (0.63 fold), while it induced the expression of cytochrome c (4.3 fold) and cleaved-caspase 9/3 (18.3/17.1 fold respectively) compared to control cells. The treatment of cells with butyrate in presence of anti-miR-22 oligos increased bcl-2 expression (2.4 fold) and decreased the expression of cytochrome c, caspase 9 and caspase 3 (1.3, 5.1 and 8.1 fold respectively) compared to butyrate alone treatment group (Fig. 5**B**). Similarly, addition of NAC along with butyrate induced bcl-2 expression (2.1 fold) and inhibited the apoptotic genes including, cytochrome c (1.8 fold), cleaved caspase 9 (6.3 fold) and cleaved caspase 3 (7.3 fold) compared to the cells treated with butyrate (Fig. 5**C**). Further, apoptosis induction was determined by Annexin V-PI assay, which showed that there was a significant increase in apoptosis with butyrate treatment, while butyrate-induced apoptosis

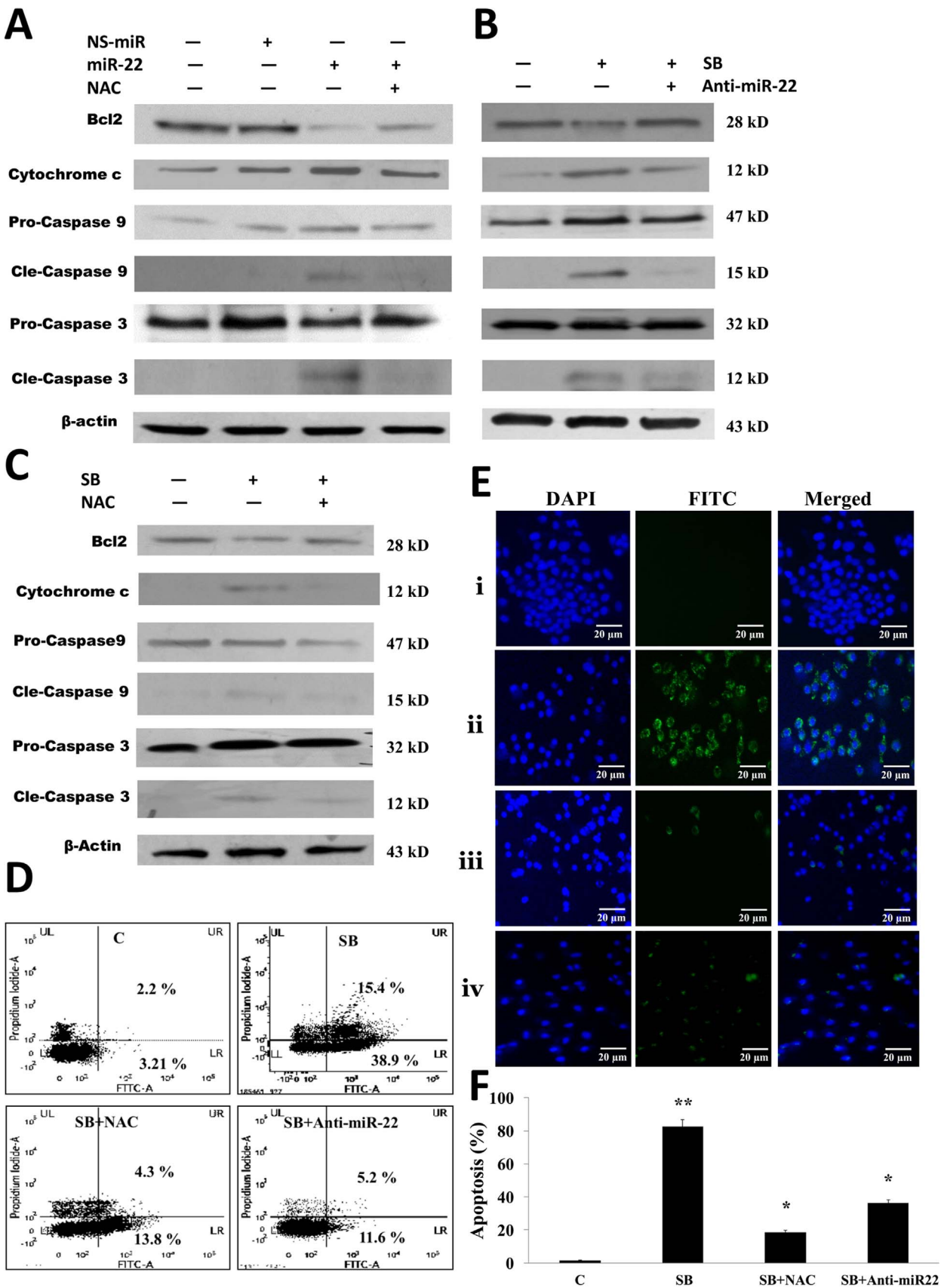

Fig. 5. Butyrate induces apoptosis via ROS production. Western blots were performed for bcl-2, cytochrome c, caspase 9, caspase 3 and β-actin in the cells incubated with **(A)** NS miR or miR-22 alone or miR-22 and NAC together (n=3); **(B)** SB alone or SB and anti-miR-22 together (n=3); and **(C)** SB alone or SB and NAC together (n=3). **(D)** Annexin V-FITC and PI staining was done in the cells treated with SB alone or SB and NAC or SB and anti-miR-22. (n=3). **(E)** TUNEL assay was performed in the cells treated with butyrate alone or SB and NAC or SB and anti-miR-22. **i,** control; **ii,** Butyrate (5 mM); **iii,** SB+NAC; and **iv,** SB+anti-miR-22. **(F)** The TUNEL positive cells were counted in at least 10 different high power fields and the percent of apoptosis was calculated (n=3; *p < 0.05; **p < 0.01).

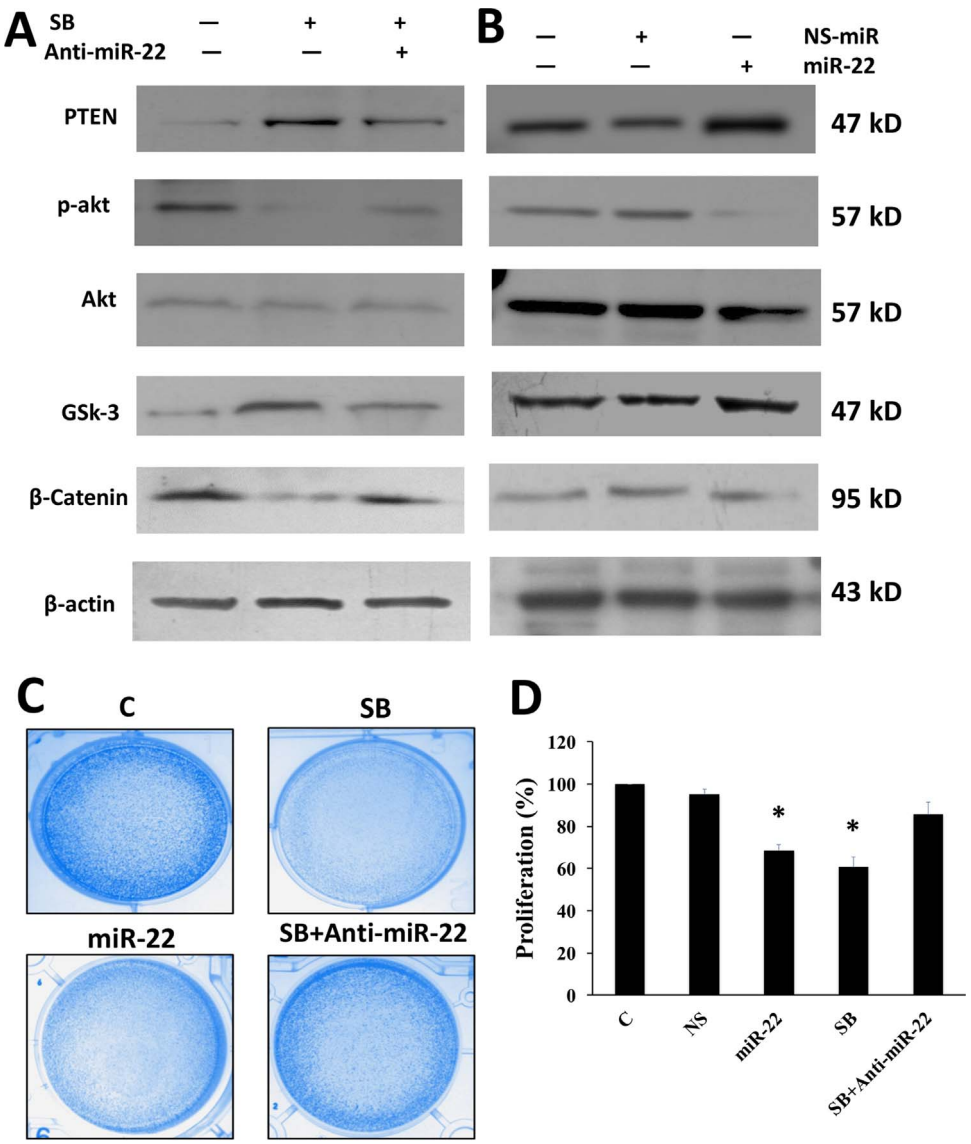

Fig. 6. Butyrate inhibits cell proliferation. Western blots were performed for p-akt, akt, gsk-3, β-catenin and β-actin in cells treated with **(A)** SB alone or SB and anti-miR-22 together; and **(B)** NS-miR or miR-22. **(C)** Colony formation assay was performed to determine the number of colonies in SB alone or miR-22 alone or SB and anti-miR-22 together. This is a representative picture from 3 experiments. **(D)** CellTiter-Blue cell viability assay was performed in cells treated with NS-miR or miR-22 alone or SB or SB and anti-miR-22. (n=3; *$p < 0.05$).

was inhibited by co-incubating with either NAC or anti-miR-22 (Fig. 5**D**). To confirm these findings, a TUNEL assay was performed and found that butyrate treated cells showed increased percentage of the TUNEL positive cells (79.1%), while addition of either NAC or anti-miR-22 inhibited the butyrate-induced cell death (28.3% & 38.4% respectively) (Fig. 5**E and F).**

3.6. Butyrate inhibits cell proliferation

Further to determine the effects of the butyrate on cell proliferation, the key proteins involved in the cancer cell growth was estimated by Western blotting. Butyrate incubation significantly decreased the levels of p-akt and β-catenin (0.1 and 0.34 fold respectively), while it increased the expression of anti-proliferative genes, PTEN and gsk-3 (5.7 fold and 4.1 fold respectively). Co-incubation of butyrate with anti-miR-22 oligos reversed the butyrate induced changes (Fig. 6**A**). The role of miR-22 was confirmed by transfecting cells with miR-22 premiR oligos and found that the changes on these proteins were similar to butyrate treatment (Fig. 6**B**). Further to confirm the effects of the butyrate on cell proliferation, the colony formation assay was performed and found that there was a decreased number of the colonies in the presence of butyrate alone or miR-22 alone. When butyrate was co-incubated with anti-miR-22, there was no significant change in the colony numbers compared to control (Fig. 6**C**). In order to substantiate these results, CellTiter-Blue cell viability assay was also performed. The data showed that the addition of butyrate or miR-22 decreased the cell proliferation to 60.6% and 68.7% respectively and addition of anti-miR-22 along with butyrate reversed the proliferation back to the normal levels (Fig. 6**D**).

3.7. Butyrate inhibits super oxide dismutase

SIRT-1 is known to regulate the SOD gene expression in order to quench the ROS production in the cells [23]. Hence, it was hypothesized that ROS-mediated apoptosis in the hepatic cancer cells occur, at least in part, by the down-regulation of the SIRT-1, which might result in the decreased levels of superoxide dismutase (SOD). The total cellular antioxidants were tested by the ABTS free radicals (a blue colour solution) scavenging assay for the presence of antioxidants. The presence of antioxidants in the ABTS decreases the intensity of the blue

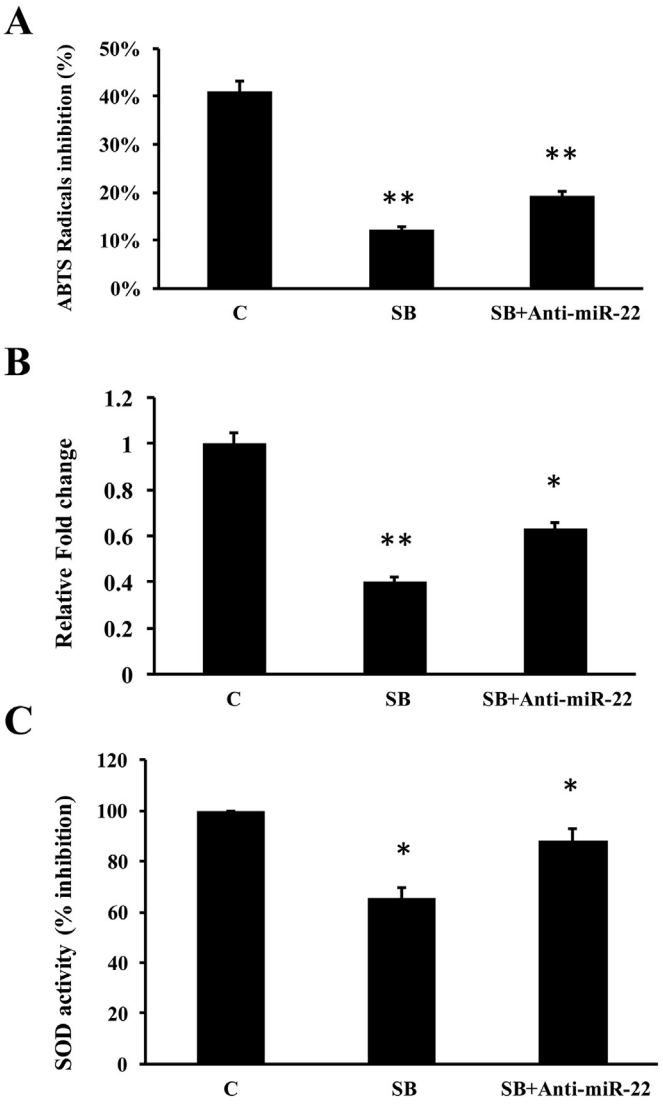

Fig. 7. Butyrate inhibits SOD expression. The cells were incubated with SB alone or SB and anti-miR-22 together. **(A)** Percentage of ABTS radicals scavenging was calculated. (n=3; **p < 0.01) **(B)** Real time RT-PCR for SOD gene expression was performed. (n=3; *p < 0.05; **p < 0.01) **(C)** SOD activity was measured and the percentage was calculated. (n=3; *p < 0.05).

colour of the solution which is measured at the 740 nm. In the butyrate-treated cells, ABTS free radicals were found to be significantly decreased (12.2%), and was reversed significantly in the presence of anti-miR-22 (Fig. 7**A**). Real time RT-PCR analysis of the SOD2 gene indicated that there was a significant decrease (0.3 fold) in butyrate treated cells and the presence of anti-miR-22 along with butyrate could significantly prevent the butyrate-mediated SOD2 gene expression (Fig. 7**B**). The SOD activity from the total cellular protein was found to be inhibited (63.8%) by butyrate treatment, while this was partially recovered (88.1%) by anti-miR-22 (Fig. 7**C**).

4. Discussion

Being an HDAC inhibitor, butyrate has been evaluated in clinical trials as a potential anticancer drug for treating human cancer [4,24,25]. Butyrate decreased tumor proliferation and induced apoptosis through intrinsic apoptosis pathway via modulation of ROS in various cancer types [4,26–29]. Although butyrate has been shown to inhibit proliferation and induce apoptosis, there is a paucity of data in the mechanisms regulating this process. To unravel the possible mechanism, the effect of butyrate on the expression of some of the key miRNAs that are shown to be involved in regulating hepatic cell functions were tested. Among the miRNAs tested, miR-22 was found to be highly modulated by butyrate treatment. Hence, it was hypothesized that the butyrate-induced miR-22 might play an important role in regulating proliferation and apoptosis in hepatic cells and selected for further experiments.

Several miRNAs including, miRNA-34a [30], miRNA-128 [31] and miRNA-204 [32] have been reported to possess the anticancer activities by targeting SIRT-1 gene. miR-22 was shown to be down-regulated in the human liver cancer tissues [33] and in other cancer cells [21,34]. The decreased miR-22 level is considered to be responsible for increased tumorigenicity, cell proliferation [33], and metastasis [35]. Over-expression of miR-22 had anti-proliferative and anti-cancer effects in various cancer cells [21,35]. In the present study, miR-22 significantly induced apoptosis and decreased cell proliferation, well correlating with previous studies. SIRT-1 was a direct target of miR-22, as miR-22 binds to the 3' UTR (530–537 bp) of SIRT-1. The butyrate also has been reported to inhibit SIRT-1 gene expression and could possibly be modulating the cancer cell proliferation and apoptosis [27,36,37]. Over-expression of miR-22 or butyrate incubation induced apoptosis and inhibited cell proliferation, while addition of anti-miR-22 along with butyrate inhibited apoptosis and induced cell proliferation. This suggests that butyrate might be mediating its effects through miR-22 mediated down-regulation of SIRT-1 expression.

SIRT-1 was shown to regulate the cellular antioxidants, like SOD, to maintain the ROS levels at physiological levels [23]. SIRT-1 expression was high in the tumor cells [38] and it increased the expression of intracellular antioxidants including SOD, thereby maintaining the anti-apoptotic [8] and pro-proliferative state [38]. SIRT-1 was also shown to be responsible for the cancer cell survival and drug resistance [11,39]. Several reports showed that SIRT-1 decreased oxidative stress by directly deacetylating several transcription factors that regulate anti-oxidant genes expression [40,41]. SIRT-1 maintains mitochondrial potential by activating glutathione peroxidase (GPx1), catalase, and SOD [23,42]. Decreased SIRT-1 expression by the chemotherapeutic drugs was reported to induce the cell death [43] inhibit tumor growth inhibition [25], furthermore the cell death was mediated by ROS production [2,44,45] and modulation of ψ_m [45]. In our study, inhibition of SIRT-1 expression by butyrate alone or miR-22 alone resulted in increased ROS production, altered ψ_m and enhanced apoptosis, while upon addition of either NAC or anti-miR-22 reversed these butyrate-mediated effects.

It was found that down-regulation of SIRT-1 by miR-22 or butyrate resulted in the inhibition of bcl2 expression, while increasing the expression of cytochrome c, caspase 9, and caspase 3, thereby inducing apoptosis. Compounds which inhibit p-akt levels by increasing ROS levels, also lead to cell death and inhibited proliferation [46,47]. When ROS was neutralized using NAC, neither butyrate nor miR-22 could induce apoptosis in hepatic cells confirming that butyrate induced miR-22, which in turn down-regulated SIRT-1 expression and enhanced ROS release. This cascade effects caused enhanced cytochrome c, caspase 3 activity and resulting increased apoptosis.

Inhibition of SIRT-1 by butyrate resulted in the inhibition of cell proliferation, whereas co-incubation with anti-miR-22 reversed the anti-proliferative effects of butyrate. The positive regulators of cell proliferation are p-akt and β-catenin expression [48,49], which are key targets for the chemotherapeutic drugs [48,50]. Our data further indicated that miR-22-mediated SIRT-1 down-regulation resulted in decreased p-akt and β-catenin expression, and increased gsk-3 and PTEN expression. Both gsk-3 and PTEN are tumor suppressor genes [51]. Thus, butyrate might negatively regulate cell proliferation via down-regulating SIRT-1, which in turn up-regulate PTEN and gsk-3 resulting in decreased p-akt and β-catenin.

The data from this study showed that butyrate induced apoptosis and inhibited cell proliferation via enhancing the intracellular expres-

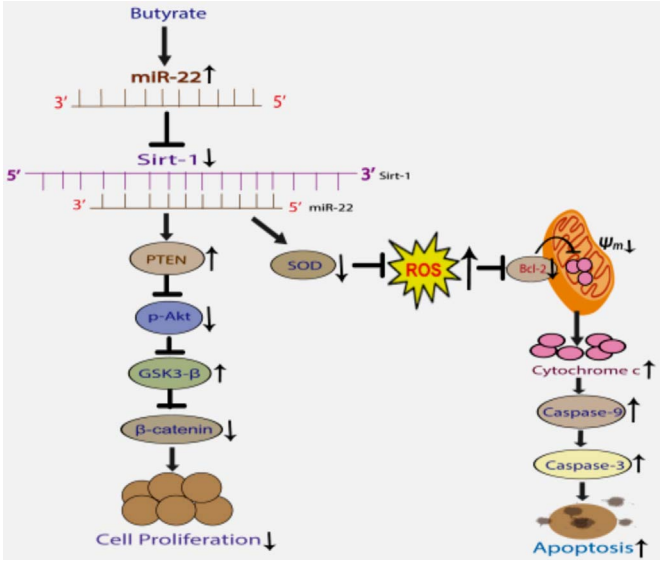

Fig. 8. The illustration shows the proposed overall mechanism by which butyrate inhibited proliferation and induced apoptosis in hepatic cells.

sion of miR-22. Further miR-22, by targeting SIRT-1, decreased cell proliferation via PTEN/p-akt pathway and increased apoptosis via ROS as SIRT-1 inhibition caused a decrease in SOD activity. The proposed pathway is presented in Fig. 8. In conclusion, we identified miR-22 is a novel regulatory molecule involved in butyrate-induced apoptosis in liver cancer cells. Hence, the present investigation provides a valuable insight mechanism that could be useful for clinical applications of butyrate to treat the hepatocellular carcinoma progression.

Competing interests

Authors declare no conflict of interest and also no financial interests are associated with this manuscript.

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Authors' contributions

KP has performed all transfection experiments, western blot, ROS analysis and most of the assays. KP also has analyzed data with the help of SKV and written the manuscript. AKY and PG were responsible for running Western blots and helped in the cell culture work. RI has done colony formation assays and did some of the assays. AS planned some of the experiments and helped in analysing the data. SKV designed the experiments, overseen the progress of work, corrected the manuscript.

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