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Original Article

Short Chain Fatty Acid Sodium Butyrate Increases miR-21, miR-143 and miR-145 Expression in Human Colorectal Cancer HCT-116 Cell Line

Parastoo Mohammadi [1], ****Flora Forouzesh*** [1], ***Fatemeh Kouhkan*** [2]

1. *Department of Genetics, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran*
2. *Stem Cell Technology Research Center, Tehran, Iran*

***Corresponding Author:** Email: f8forouzesh@gmail.com

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Abstract

Background: Sodium butyrate (NaBu) is a short-chain fatty acid; it is one of the histone deacetylase inhibitors, which can alter both genetic and epigenetic expressions. The present study aimed to elucidate the effect of Na-Bu on the expression of miR-21, miR-143, and miR-145 in human colorectal cancer HCT-116 cell lines.

Methods: This study was done in Tehran Medical Sciences, Islamic Azad University, Tehran, Iran. HCT-116 cell line was treated with diverse concentrations of NaBu (6.25 mM to 200 mM) at 24, 48, and 72 h. MTT assay was used for assessing the cytotoxicity. Quantitative Real-Time-PCR was performed to investigate the gene expression of miR-21, miR-143, and miR-145.

Results: IC50 values were evaluated by MTT assay. IC50 for HCT-116 was 50 mM, 12.5 mM, and 6.25 mM for 24, 48, and 72 h of incubation, respectively. According to the Real-Time-PCR results, 50 mM NaBu after 24 h caused a significant up-regulation in the expression of the miR-21, miR-143, and miR-145 ($P<0.05$). In 48 h, incubation, 12.5 mM NaBu caused a significant up-regulation in the expression of the miR-21, miR-143, and miR-145 ($P<0.05$). In treated cells with 6.25 mM NaBu after 72 h of incubation caused a significant up-regulation in the expression of the miR-21, miR-143, and miR-145 compared with untreated cells ($P<0.05$).

Conclusion: The upregulation of miR-21, miR-143, and miR-145 expression are mediated by transcriptional regulation and the activation of this miR promoter is modulated by histone acetylation. The employment of NaBu may represent a promising approach for improving HDACi drug-based therapies for colon cancers.

Keywords: Colorectal cancer; Sodium butyrate; Human miR-21; Human miR-143; Human miR-145; Human HCT-116 cell

Introduction

Colorectal carcinoma (CRC) is the most common tumor affecting the digestive tract. According GLOBOCAN 2012 data, CRC is the second common cancer in women and the third common cancer in men (1) leading to mortality.

One of the pathways for treating cancer is controlling the epigenetics pathways (2). Epigenetics mechanism such as MicroRNAs (3) are significantly involved in cellular proliferation, differentiation, and apoptosis (4), thus, they are signifi-

cant in cancer inhibition and progression. MicroRNA expression is deregulated in cancer processes including cancer initiation, progression, and metastasis (5). They are promising targets in the treatment and diagnosis of CRC. Accordingly, some of MicroRNAs have decreased expression and some of them increased expression in CRC represents the first step of this process (6).

The cluster of miR-143 and miR-145 with various roles in various different kinds of cellular functions are co-expressed in many cell types and tissues (7). These two miRs have similar functions in most cells. They are expressed in normal tissues at significant levels, with highest expression in colon and lowest in brain and liver (7). The level of expression of these miRNAs is significantly high in stomach, small intestine, prostate, cervix, uterus; their expression level is low in kidney, spleen, placenta, testis, and skeletal muscle (7). Both miR-143 and miR-145 are reduced in various types of cancers, such as hematopoietic system, lung, colon, the gastrointestinal system, breast, prostate, ovary, cervix, bladder, head and neck (8, 9).

miR-21 is an oncomir and its expression increases in the majority of solid tumors including prostate (10), colon (11), lung cancer (12), and etc. More importantly, the level of expression of miR-21 is consistently associated with established prognostic factors constituting a significant predictor of survival in CRC (13) and in non-small-cell lung cancer (12). The most common anticancer drugs that cause epigenetics modification in tumor cells are histone deacetylase inhibitors (HDACi) (14). HDACi are used as chromatin-modifying drugs for treating cancers and other diseases (15). Sodium butyrate (NaBu) can act as HDACi to promote human health (16).

NaBu is a short chain fatty acid (SCFAs), and it is produced naturally, during the process of microbial fermentation fibers in the colon (17). NaBu is involved in regulating the expression of microRNAs for inhibiting the proliferation of colorectal cancer cells (18).

The aim of present study was investigating the effect of NaBu on miR-21, miR-143 and miR-145 expression in human colorectal cancer HCT-116 cell line.

Materials and Methods

This study was approved by the ethics committee of the Azad Islamic University of Medical Sciences. Ethics review report number is IR.IAU.PS.REC.1397.091.

Cell culture

This study was done in Tehran Medical Sciences, Islamic Azad University, Tehran, Iran. HCT-116 human colorectal cell line was purchased from Pasteur Institute of Iran (Tehran, Iran). HCT-116 cells was cultured in Dulbecco's Modified Eagle Medium plus GLUTAMAX (DMEM+GLUTAMAX) (Low Glucose) (Gibco, Germany) supplemented with 10% fetal bovine serum (FBS) (Gibco, Germany) and antibiotics (100,000 U/Lpenicillin and 100 mg/L streptomycin), Gibco, Germany) at 37°C and 5% CO2. Monolayer cells were harvested by 0.25% trypsin-EDTA (Gibco, Germany). Overall, 50×10^3 cells per well (the optimized cell number) were seeded in 96-well plates and incubated for 24 h (19). HCT-116 cells were treated with different concentrations of NaBu (Sigma, USA) (6.25 mM, 12.5 mM, 25 mM, 50 mM, 100 mM, 150 mM and 200 Mm) at 24, 48, and 72 h. Untreated cells (0 mM) and treated cells with dimethyl sulfoxide (DMSO) 20% were set as negative and positive controls, in the respective order.

Cytotoxicity assay

The cytotoxicity effect of NaBu in HCT-116 colorectal cancer cell line was studied by 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazo¬lium bromide (MTT) assay (Sigma, USA) after 24, 48 and 72 h of treatment and was compared with the untreated cells (0 Mm) as a control group, then added 100 µl of MTT solution to each well and incubated at 37 °C and 5% CO2 depending on the dehydration activity for 3 to 4 h. The formazan crystals were formed and dissolved in 50 µl DMSO and the absorbance was measured

at 546 nm using an ELISA reader (Biotek, USA). Ultimately, IC50 (the concentration of 50 % of the cells is alive) is calculated. Cell viability rate (%) =(OD546 of treated cells/OD546 of control cells)×100 %.

RNA extraction and cDNA synthesis

RNAXPLUS (Sinaclon, Iran) was applied according to the manufacturer's instructions for total RNAs extraction. All RNA related operations were carried out under RNase-free conditions. RNA concentration was determined through a Biophotometer plus (Eppendorf, Germany) at 260 nm to 280 nm and 1% agarose gel electrophoresis for confirming the integrity of total RNA, stored at -80 °C. 2000 ng of total RNA extracted from cells was reverse transcribed to cDNA with the hexamer and M-MLV RT kit (Yektatajhiz, Iran).

Quantitative Real-Time polymerase chain reaction (qRT-PCR)

The expression level of miR-21, miR-143, and miR-145 was quantified by quantitative real-time PCR, which was performed using the Applied Biosystems 7500 Sequence Detection System (Applied Biosystems-Germany). SYBR green-based quantitative real-time PCR analysis was performed). The expression of miRNA was defined by the threshold cycle (Ct), and relative expression levels were calculated after normalization with reference to expression of snord-47 (endogenous control).

Statistical analysis

GraphPad Prism was used to carry out all statistical analyses. Data are represented as means ± SD of three independent experiments, each performed in triplicate. Statistical significance between treatment and control groups was analyzed using the one-way ANOVA. A value of $P<0.05$ was considered statistically significant.

Results

Cytotoxicity assay

The cytotoxicity effect of NaBu on cell viability of the HCT-116 cell line was investigated through the MTT assay at different concentrations of NaBu (0 mM to 200 mM) at 24, 48, and 72 h (Fig.1). IC50 (the concentration of 50 % of the cells is alive) concentration of NaBu at 24 h was less than 50 mM (between 25 mM and 50 mM), it was at 48 h less than 12.5 mM (between 6.25 mM and 12.5), and IC50 concentration at 72 h was almost 6.25mM. The growth of the HCT-116 cell line was inhibited by NaBu in a dose-dependent manner.

NaBu upregulates miR-21 in human colorectal cancer HCT-116 cells

The expression of the miR-21 gene in HCT-116 cell line treated based on IC50 concentration of NaBu and the concentration after IC50 concentration, 25 mM, 50 mM, and 100 mM in 24 h, 12.5 mM, 25 mM and 50 mM in 48 h and 6.25 mM, 12.5 mM and 25 mM in 72h revealed distinct gene expression profiles in comparison with untreated cells. After NaBu treatment with 25 mM, 50 mM, and 100 mM concentration within 24 h incubation, miR-21 was upregulated significantly ($P<0.05$) and more than 12.3-fold, 2-fold and 24.2-fold respectively compared to untreated cells (0 mM) (Fig.2, A).

After NaBu treatment with 12.5 mM, 25 mM and 50 mM concentration within 48 h incubation, miR-21 was upregulated significantly ($P<0.05$) and more than 4.8-fold, 8.9-fold and 11.7-fold compared to untreated cells (0 mM) (Fig.2, B).

After NaBu treatment with 6.25 mM, 12.5 mM and 25 mM concentration in 72 h incubation, miR-21 was upregulated significantly ($P<0.05$) and more than 3.8-fold, 4.3-fold and 3-fold compared to untreated cells (0 mM) (Fig.2, C).

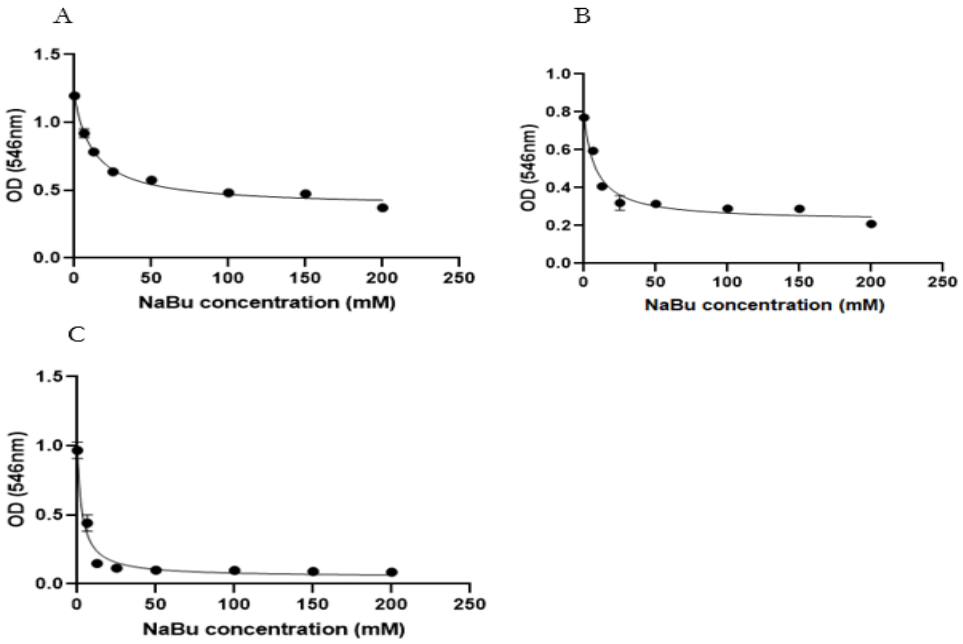


Fig.1: The dose-response effect of NaBu on cell viability. Human colorectal cancer HCT-116 cell line (10^6 cells/mL) was incubated at 37 °C for A) 24 h, B) 48 h and C) 72 h following the addition of different concentrations of NaBu (0 mM, 6.25 mM, 12.5 mM, 25 mM, 50 mM, 150 mM and 200 mM); cytotoxicity was assessed using MTT assays

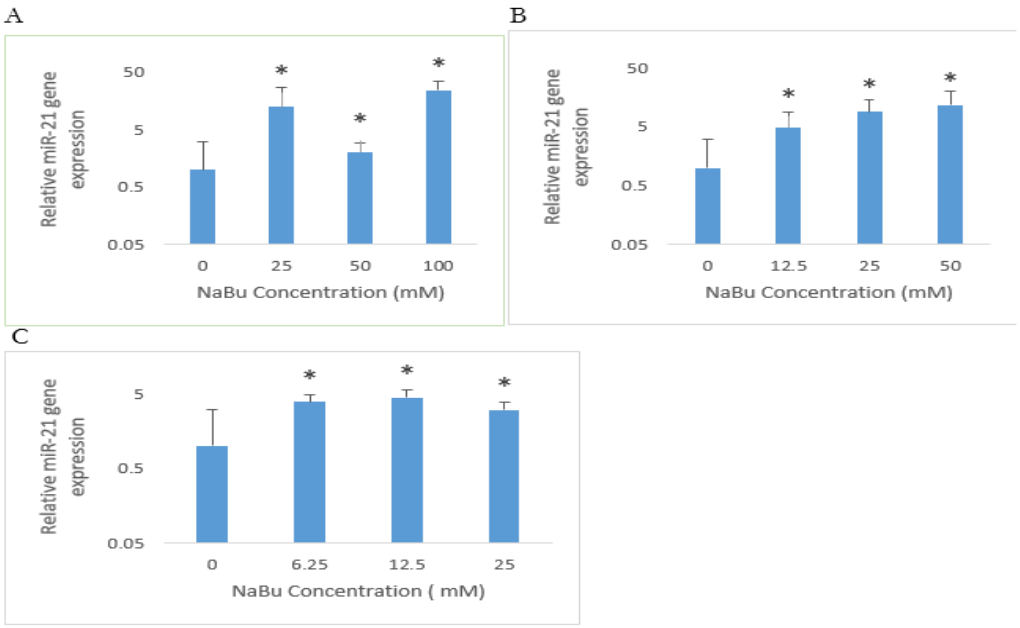


Fig.2: Expression mRNA levels of the miR-21 in HCT-116 NaBu -threated after 24, 48, and 72 h incubation in comparison with HCT-116 untreated cells. A) miR-21 expression was upregulated at 25 mM, 50 mM, and 100 mM concentrations after 24 h treatment with NaBu. B) After 48 h treatment with NaBu, miR-21 expression was increased at 12.5 mM, 25 mM and 50 mM concentrations in NaBu-treated HCT-116 cell compared with untreated group. C) After 72 h treatment with NaBu, miR-21 expression was increased at 6.25 mM, 12.5 mM and 25 mM concentrations as compared to the untreated control group. The values represent the means with standard deviations (SD) of the expression levels of gene. *Significant reduction in mRNA expression at $P<0.05$ compared with untreated cells

NaBu upregulates miR-143 in human colorectal cancer HCT-116 cells

To determine the effect of NaBu treatment on miR-143 expression in HCT-116 cell line, HCT-116 cells were treated based on IC50 concentration. They were treated with 25 mM, 50 mM, and 100 mM in 24 h, 12.5 mM, 25 mM and 50 mM in 48 h and 6.25 mM, 12.5 mM and 25 mM in 72h. The expression of miR-143 was significantly upregulated in untreated HCT-116 cells and NaBu-treated groups.

After NaBu treatment with 25 mM, 50 mM, and 100 mM concentration in 24 h incubation, miR-143 was upregulated significantly (P<0.05) and more than 14.1-fold, 5-fold and 9.3-fold respectively when compared with untreated HCT-116 cells (0 mM) (Fig.3, A).

After NaBu treatment with 12.5 mM, 25 mM and 50 mM concentration in 48 h incubation, miR-143 was upregulated significantly (P<0.05) and more than 2.2-fold, 4.8-fold and 3.7-fold when compared with untreated HCT-116 cells (0 mM). (Fig.3, B).

After NaBu treatment with 6.25 mM, 12.5 mM and 25 mM concentration in 72 h incubation, miR-143 was upregulated significantly (P<0.05) and more than 2.64-fold, 7.8-fold and 7.7-fold when compared with untreated HCT-116 cells (0 mM) (Fig.3, C).

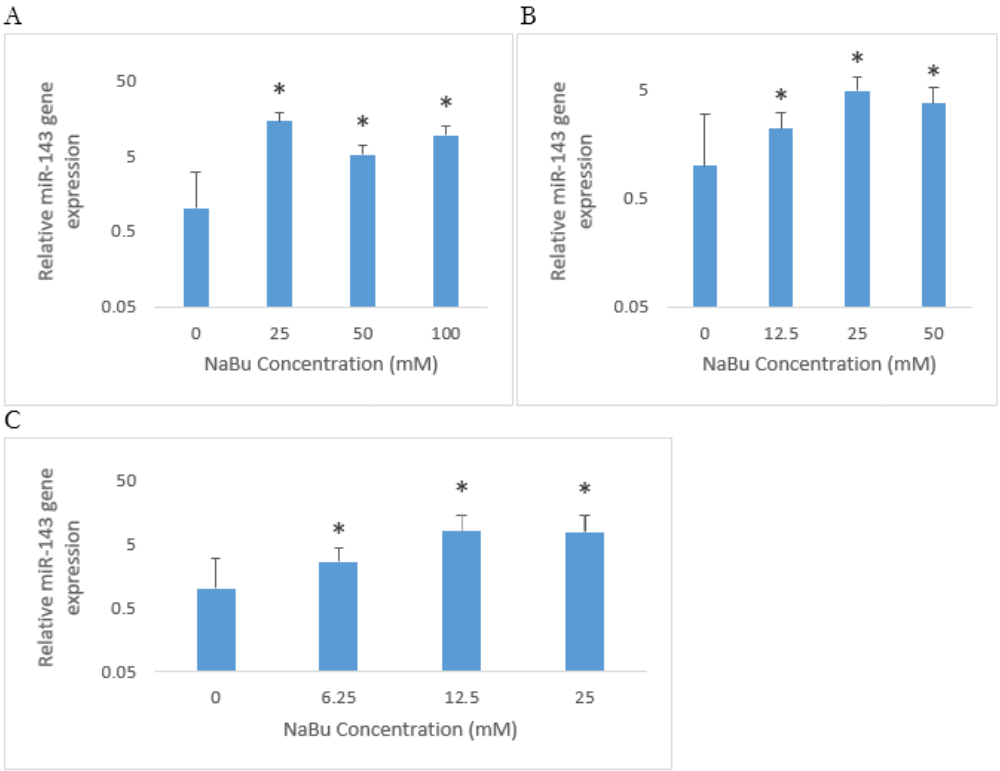


Fig.3: Expression mRNA levels of the miR-143 in HCT-116 NaBu -threated after 24 h, 48 h and 72 h incubation in comparison with HCT-116 untreated cells. A) miR-143 expression was upregulated at 25 mM, 50 mM, and 100 mM concentrations after 24 h treatment with NaBu. B) After 48 h treatment with NaBu, miR-143 expression was increased at 12.5 mM, 25 mM and 50 mM concentrations in NaBu-treated HCT-116 cell as compared with untreated group. C) After 72 h treatment with NaBu, miR-143 expression was increased at 6.25 mM, 12.5 mM and 25 mM concentrations as compared to the untreated control group. The values represent the means with standard deviations (SD) of the expression levels of gene. *Significant reduction in mRNA expression at P<0.05 compared with untreated cells

NaBu upregulates miR-145 in human colorectal cancer HCT-116 cells

RT-qPCR was used to investigate differentially expressed miR-145 in untreated cells as control group and NaBu -treated HCT-116 cells. HCT-116 cells were treated based on IC50 concentration. They were treated with 25 mM, 50 mM, and 100 mM in 24 h, 12.5 mM, 25 mM and 50 mM in 48 h and 6.25 mM, 12.5 mM and 25 mM in 72h. The expression of miR-145 was significantly upregulated in control and NaBu-treated groups.

After NaBu treatment with 25 mM, 50 mM, and 100 mM concentration in 24 h incubation, miR-145 was upregulated significantly (P<0.05) and more than 4-fold, 2-fold and 7.6-fold respectively when compared with them in the control group (0 mM) (Fig.4, A).

After NaBu treatment with 12.5 mM, 25 mM and 50 mM concentration in 48 h incubation, miR-145 was upregulated significantly (P<0.05) and more than 4.5-fold, 8.2-fold and 4.4-fold when compared with them in the control group (Fig.4, B).

After NaBu treatment with 6.25 mM, 12.5 mM and 25 mM concentration in 72 h incubation, miR-145 was upregulated significantly (P<0.05) and more than 4.3-fold, 4.7-fold and 10.7-fold when compared with them in the control group (0 mM) (Fig.4, C).

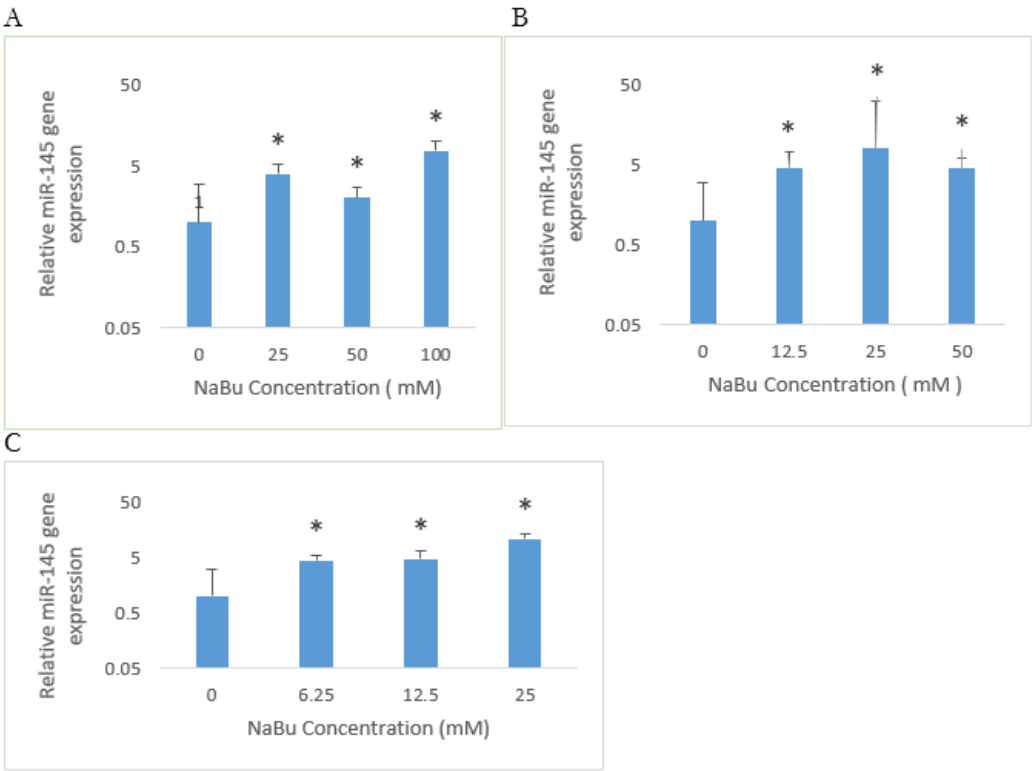


Fig.4: Expression mRNA levels of the miR-145 in HCT-116 NaBu -threated after 24 h, 48 h and 72 h incubation in comparison with HCT-116 untreated cells. A) miR-145 expression was upregulated at 25 mM, 50 mM, and 100 mM concentrations after 24 h treatment with NaBu. B) After 48 h treatment with NaBu, miR-145 expression was increased at 12.5 mM, 25 mM and 50 mM concentrations in NaBu-treated HCT-116 cell as compared with untreated group. C) After 72 h treatment with NaBu, miR-145 expression was increased at 6.25 mM, 12.5 mM and 25 mM concentrations as compared to the untreated control group. The values represent the means with standard deviations (SD) of the expression levels of gene. * Significant reduction in mRNA expression at P<0.05 compared with untreated cells

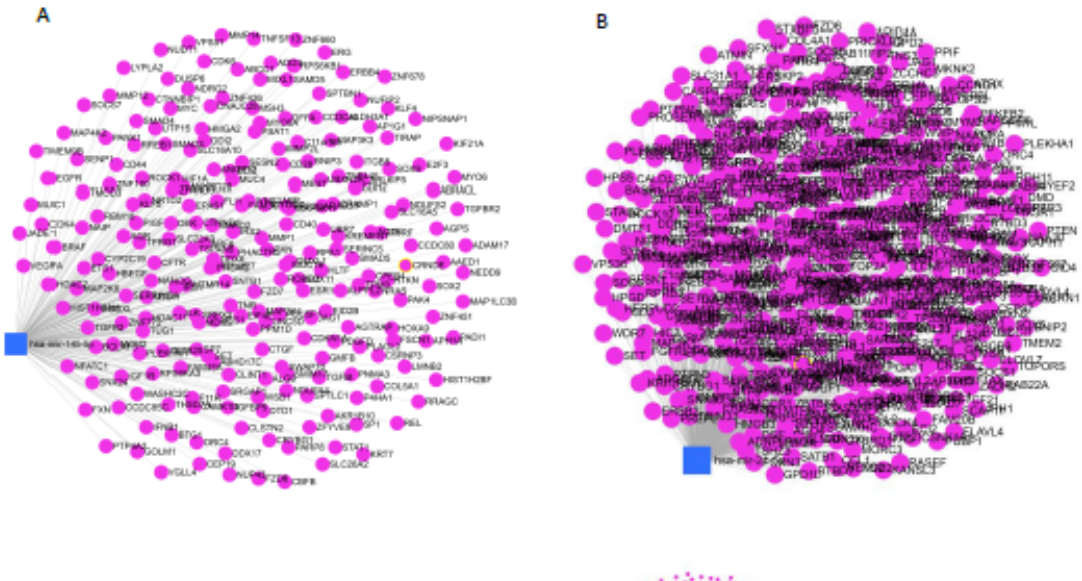
Discussion

Our results showed that NaBu increased miR-21, miR-143, and miR-145 in treated HCT-116 cell line. Preventable of CRC disease has always attracted the attention of health centers around the world. Despite the significant role of inheritance, most cases of CRC have been sporadic and they develop slowly over several years. Screening can significantly reduce the incidence and mortality of this cancer (20).

MicroRNAs are epigenetically capable of regulating gene expression by post-transcriptionally regulating messenger RNA (mRNA) function, which can inhibit or promote protein production. MicroRNAs are commonly present in both normal and malignant cells and they are capable of regulating at least 30% of human genes (21). Aberrant miRNA expression is linked with CRC initiation, progression, and metastasis (22). Furthermore, epigenetics drugs, such as histone deacetylation (4-phenyle butyric acid) inhibitors, increase the expression of miRs, by reducing the DNA methylation and increasing histone acetylation, and preventing cell proliferation (23). The butyrate as HDACi can modulate apoptosis, cell proliferation, differentiation, and miRNA expression in CRC (22). HDACi have been investigated in clinical trials with promising results regarding safety and toxicity (24).

miR-21 is located on chromosome 17 and it increases the expression in CRC. It acts as an oncogene (25). This paper focused on the effect of NaBu on the expression of miR-21 in HCT-116 cell line. Contrary to the expectations, our results revealed that NaBu increased the expression of miR-21 in treaded cell compared with control group. Probably, NaBu by increasing the expression of miR-21 in the treated cell line caused the target genes of miR-21 impressed and inducing apoptosis in these cells. miR-21 targets other genes to exert its effect. This finding is in agreement with previous findings. AS2O3 in promyelocytic leukemia inhibited the expression of the SP1 target gene and the HERG canal by increasing the expression of miR-21 in the HERG-HEK293 cell line (26). NaBu decreased the expression of miR-17-92a unlike miR-21 in both HCT-116 and HT-29 cell line. miR-17-92a like miR-21 showed increased expression in CRC. Treating human colon cancer cells with butyrate reduced the levels of pri-miR17-92a (27). The cluster of 143/145 miRNA is located on chromosome 5 in human and it inhibits cell proliferation and inhibits invasion and migration (28). miR-145 inhibits tumor cell proliferation, invasion, and metastasis (29, 30). It regulates tumor occurrence and development. This distinctive role of miR-145 in the regulation of metastasis-related gene expression may introduce miR-145 as an ideal candidate for controlling cancer metastasis by miRNA replacement therapy (31). The cluster of 143/145 is down regulated in CRC and many types of cancers (32-34). miR-145 is a potential marker for the early diagnosis and prognostic evaluation of patients with cancer; it acts as a tumor suppressor, and it is believed to be a promising cancer treatment target candidate (35). Sloby et al. studied the expression of miR-21, miR-31, miR-143, and miR-145 in colon cancer cells. The expression of miR-143 and miR-145 were decrease, but the expression of miR-21 and miR-31 were increase. miR-21 was positively associated with lymph node involvement and metastatic progression (36). The expression of miR-143 in the HTC-116 and SW-480 cellular cell lines examined and reported the expression of miR-143 was increased and associated with colorectal cancer metastasis. miR-143 inhibits colorectal cancer cell invasion, but it has no effect on cell proliferation (37).

Our results showed that the expression of cluster 143/145 in NaBu-treated HCT-116 cell lines was increased compared with untreated cells. Similar to our results, Han R et al. studied the effect of NaBu on the expression of miR-203 in Caco2 and HT-29 cell lines. The expression of miR-203 was increased in NaBu-treated Caco2 and HT-29 compared with untreated cells. Increasing the expression of miR-203 induced apoptosis and inhibited cell proliferation and colony formation and cellular invasion (18).



JAG1	HOXA9	SOX2	FAM3C	SLC16A10
CRYBG1	IGF1R	SP1	IVNS1ABP	SAMD5
KLF5	ITGB8	TGFB2	ANKRD28	HIF1A
CDK6	MUC1	TGFBI	WSB1	SET
SLC26A2	MYC	TGFBR2	NUFIP2	PHACTR2
E2F3	ORC4	VEGFA	RRAGC	PDGFD
EGFR	RPS6KA3	FZD6	JADE1	

Fig.5: Schematic plot of the miR-145 and miR-21-genes interactions based on miRNet. The blue square represents the miRNA and Purple nodes represent target genes. A: Genes that are targeted by miR-145. B: Genes that are targeted by miR-21. C: The shared nodes between "miR-145" and "miR-21". 34 genes targeted by both "miR-145" and "miR-21" are highlighted in the box and the list of these genes showed in the table

To investigate the interactions between cluster of miR-145/143 and miR-21 and their target genes, we used the miRNet tool which contains miRNA–gene interaction data collected from miRTarBase v7.0, TarBase v7.0 (Fig. 5). According to the miRNet miR-145 and miR-21 have interaction with different genes separately (Fig. 5-A, B). Moreover, we determined the shared target

genes that miR-145 and miR-21 interacted with them together (Fig. 5-C). 34 genes targeted by both "miR-145" and "miR-21" are highlighted in the box. These genes have main roles in important cellular pathways. The interplays between miRNAs and target genes show only the starting points to understanding the roles that miRNAs play at the cellular level. In specific, miRNAs can control gene regulatory networks via feedforward loops or feedback. Therefore, it can be reasonable to presume both "miR-145" and "miR-21" might be good candidates for further extensive studies on cancer and targeted therapy.

Conclusion

NaBu is capable of upregulating miR-21, miR-143 and miR-145 to inhibit cell growth and promote apoptosis in HCT-116 cells in a time- and concentration-dependent manner. NaBu is an HDACi that can change histones and they in turn can modulate the expression of these miRNAs in colon. The cluster of miR-145/143 as a tumor suppressor gene and miR-21 as oncomir can be considered as candidates for cancer treatment. These miRNAs, by regulating multiple targets, can exert potent effects on cancer cell growth and tumorigenesis suggesting a potential new therapeutic strategy in colorectal cancer in the future; however, more studies are needed.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflicts of Interest

The authors declare no conflicts of interest.

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