

Effect of Sodium Butyrate on Epithelial-Mesenchymal transformation in colorectal cancer cell line SW620

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Abstract: Butyric acid is produced by endogenous intestinal bacteria during the fermentation of dietary fiber, and has been found to have anticancer effects. The aim of the present study was to identify that if sodium butyrate (NaB) affects proliferation, migration, invasion, adhesion abilities and epithelial-mesenchymal transition (EMT) in CRC cells (SW620), and explore its possible mechanisms. In this study SW620 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum in a 5% CO₂ incubator at 37°C. SW620 cells were divided into 5 groups: control group, 1.25mM NaB group, 2.5mM NaB group, 5mM NaB group and 10 mM NaB group. The effect of NaB treatment on SW620 cells proliferation was detected by CCK-8 assay. The functional role of NaB on SW620 cells motility was evaluated by wound healing assay and transwell migration assay. In addition, a Matrigel invasion assay was performed to characterize the invasion ability. The adhesion ability between cells and extracellular matrix was examined by cell-interstitial adhesion test. The protein expression level of TGF- β , E-Cadherin and Vimentin were measured by Western-Blot. The results demonstrated that after treated by different concentrations (1.25mM, 2.5 mM, 5mM,10mM) of NaB for 24h, the proliferation rates of SW620 cells were inhibited significantly ($P<0.05$). Compared to the control group, the abilities of migration, invasion and adhesion of SW620 cells lines in other groups were gradually decreased, respectively ($P<0.05$). Western-Blot assay showed that NaB could significantly inhibit the expression of TGF- β and Vimentin protein and promote the expression of E-Cadherin in SW620 cells ($P<0.05$). There was a dose-effect relationship between the concentration and effect of NaB on SW620 cells in all the above experiments. These findings indicate that NaB can inhibit the abilities of migration, invasion and adhesion of SW620 cells. In addition, NaB can inhibit the EMT of CRC cells by down-regulating the expression of TGF- β and Vimentin protein and up-regulating the expression of E-Cadherin protein.

Keywords: Sodium butyrate; Epithelial-mesenchymal transformation; Colorectal cancer

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1. Introduction

CRC is the third most common cancer all over the world, meanwhile, CRC is the second leading cause of cancer mortality in the world[1]. The high mortality of CRC is closely related to the invasion and metastasis of CRC. Once invasion and metastasis occur in patients with CRC, the mortality rate of patients will increase significantly. EMT is an initial step and a major phenotype of cancer metastasis and invasion[2]. The epithelial cells lose their features and gain mesenchymal properties, which means that the adhesion between epithelial cells is weakened, the arrangement of tumor cells is loose, and most of the cells have slender pseudopodia, which can obtain the ability of migration and invasion. The transition of cells from epithelial phenotype to stromal phenotype means that the cell has an EMT process[3]. When a tumor develops EMT, tumor cells can obtain stem cell-like properties, leading to chemotherapy resistance, tumor angiogenesis and distant metastasis. EMT has a great impact on the prognosis of tumor, and is expected

to become an important target of tumor therapy[4,5]. The EMT is characterized by the combined loss of epithelial cell junction proteins, such as E-cadherin, and the gain of mesenchymal markers, such as Vimentin[6]. Transforming growth factor β (TGF- β) is a key factor in inducing EMT in tumor cells. Several studies have shown that the enhancement of TGF- β signal is a key effector of cancer invasion and metastasis. Increased production of activated TGF- β is frequently observed in cancer cells[7]. And in vivo studies found that TGF- β receptor inhibition could completely inhibit the metastasis of highly metastatic cells[8]. Therefore, TGF- β , E-cadherin and Vimentin are commonly used as marker proteins for EMT. Therefore, seeking a substance to effectively inhibit the EMT of CRC is a direction to inhibit the CRC invasion and metastasis.

NaB, as the main energy source of colonic mucosal cells, is a non-toxic short-chain fatty acid produced by dietary fiber fermentation in the intestinal tract. According to previous studies, NaB has an inhibitory effect on the proliferation and metastasis of a variety of

malignant cells, but the precise mechanism underlying this effect is unclear[9,10]. NaB could inhibit the EMT process of SW480 in human CRC cells by inhibiting hypoxia inducible factor- α in vitro[11]. Xu Z also found that NaB has inhibitory effect on invasion and metastasis of CRC by down-regulating Bim-1 and enhancing miR-200c expression[12]. Some clinical studies have found that NaB has a certain therapeutic effect on leukemia and endometrial cancer[13,14]. These findings prompted us to study the effect of NaB on the process of EMT in CRC cells. The purpose of this study is tantamount to investigate whether NaB can inhibit the invasion and metastasis of CRC by regulating the EMT process, thereby reducing the metastasis rate of CRC.

2. Materials and methods

2.1. Materials

SW620 cells were purchased from Shanghai Cell Room of Chinese Academy of Sciences; NaB from McLean Company; Matrigel matrix glue from BD Corporation; Transwell chamber from Corning costar Company; penicillin-streptomycin mixture from Solarbio Company; Western-blot experiment related reagents purchased by Beyotime Biotechnology Co. Ltd.

2.2. Cell cultures

SW620 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum, 1% penicillin and 1% streptomycin, and cultured in 5% incubator at 37 °C.

2.3. CCK-8 assay to detect the effect of NaB on cell proliferation

SW620 cells in logarithmic growth phase were inoculated into 96-well plate with 10^5 cells/well, and then intervened after the cells were attached to the wall. The final intervention concentration of the intervention group were 1.25mM, 2.5mM, 5mM, 10mM. The same volume of 1640 culture medium containing 10% FBS was added into control group. Each group was set up three duplicate holes. All the 96-well plates were cultured at 37 °C under 5% CO₂ condition. 48h later, 10 μ L CCK-8 reagent was added into each well. The absorbance value was obtained by colorimetry at 450nm on the microtiter after continuous culture for 4h.

Inhibition rate = (OD value of control group - OD value of intervention group) /

$$\text{OD value of control group} \times 100 \%$$

2.4. Wound healing test to observe the effect of NaB on cell migration

SW620 cells with 1×10^6 cells/ mL were seeded in 6-well plate and cultured in complete medium

containing 10% FBS for 24h until the fusion degree was over 90%. The cells were streaked in parallel in a 6-well plate through a 20-well micro-liquid transfer gun. The cells were washed 3 times with PBS and the floating cells were removed. The serum-free medium was added into control group, and the serum-free medium containing the corresponding concentration of sodium butyrate was added into intervention groups. The cell migration process of each group was observed under an optical microscope. Three replicas were set up in each group, and the migration of cells on both sides of the scratch was observed under an inverted microscope at 6h, 12h, 24h and 48h after the intervention, respectively. The number of cells on both sides of the scratch was measured by Image J software.

2.5. Transwell chamber assay to investigate the effect of NaB on cell migration

SW620 cells were inoculated on polycarbonate microporous filter membrane in the culture chamber by placing the culture chamber with 8 μ m microporous polycarbonate membrane in 24 culture plates. The control group was cultured in serum-free medium and the intervention group was cultured in 1.25mM, 2.5mM, 5mM and 10mM for 24h. The membrane was fixed with methanol for 0.5h and stained with 0.1% crystal violet for 0.5h.

2.6. Matrigel invasion assay to test the effect of NaB on cell invasiveness

The matrix glue and RPMI 1640 were diluted in 1:8 (this process was operated on ice). The diluted solution (50 μ L) was evenly distributed in the bottom of the upper chamber, incubated at 37 °C for overnight, and then solidified. After the three cells were digested and centrifuged, the SW620 cells concentration was adjusted to 4×10^5 . 200 μ L single cell suspension was added into each chamber and the number of cells in the chamber was 8×10^4 cells/well. NaB was added to the intervention group to make the final concentration of 1.25mM, 2.5mM, 5mM, 10mM in the upper compartment, while the serum-free medium was added into control group. 1640 medium containing 10% FBS was added into the lower chamber, and three repeats were set up in each group. After culturing at 37 °C for 48 h under 5% CO₂ condition, the cells were removed and the culture medium was discarded. The cells in the upper layer were fixed with methanol for 0.5h and stained with 0.1% crystal violet for 0.5h with cotton swabs. The images were collected from five visual fields randomly under the microscope.

2.7. Cell-matrix adhesion assay to determine the effect of NaB on cell adhesion

SW620 cells in the intervention group were treated with 1.25mM, 2.5mM, 5mM and 10mM NaB for 24h,

and normal culture in the control group. 50 μ L Matrigel matrix adhesives (2.5mg/mL) were added to each well of the plate, which was placed overnight in the incubator and blocked for 2h by 0.2% BSA. The number of cells was adjusted to a concentration of about 1×10^6 cells/ mL, and the cells were resuspended in 1640 culture medium without drugs. The cells were cultured in 3multiple holes for 1.5h in each concentration and then the culture medium was sucked out. The sterilized PBS was carefully cleaned three times and then the serum-free medium containing 100 μ L and CCK-8 reagent 10 μ L were added into each hole. After being set aside for 4h at 37 $^{\circ}$ C, the OD value was measured under the wavelength of 450nm.

2.8. Western-blot to examine related protein expression

The cells were added into RIPA lysate containing phosphatase inhibitor, and the total protein of the cells was extracted. The protein concentration was determined by BCA protein assay. At the end of the assay, the sample buffer was added to each protein, and the mixture was heated to denaturalize the protein. After the protein was cooled, it was added to the SDS-PAGE gel for electrophoresis separation, and the separated protein molecule was transferred to the PVDF membrane by wet transfer. After being sealed with TBST containing skimmed milk powder for 2h, rinsed for 3 times, the corresponding primary resistance of the target protein was added to incubate for 3h and 4h, and the second antibody was added again after rinse for 2h. The ECL luminescent solution was added into the FusionFX5 developer to obtain the target protein band. The relative expression of the protein was expressed by the ratio of the target protein to the gray value of the internal reference. The experiment was repeated 3 times and the results were effective.

2.9. Statistical Analysis

The experimental results were statistically analyzed by SPSS 18.0 software package. The experimental data were expressed as mean \pm standard deviation ($\bar{x} \pm s$). One-way ANOVA and LSD-t test were used to compare the data between the two groups. When $P < 0.05$, the difference is statistically significant.

3. Results

3.1. Different concentrations of NaB could inhibit the proliferation ability of SW620 cells

The results of cck8 assay showed that the proliferation of SW620 cells were significantly inhibited by different concentrations of NaB. As can be seen from Figure 1, the OD value decreased after the intervention of NaB. The levels of OD in 1.25mM, 2.5mM, 5mM and 10mM NaB groups were significantly lower than those in the control group ($P < 0.05$), and there were significant differences among the intervention groups. The inhibition rates of each group were $9.23 \pm 6.15\%$, $25.86 \pm 5.58\%$, $49.47 \pm 1.26\%$, $72.21 \pm 1.39\%$.

3.2. Different concentrations of NaB could control the migration ability of SW620 cells

The wound-healing assay is a simple and effective method to detect tumor cell migration. It can be seen from the wound healing experiment that with the increase of intervention time and concentration, the inhibitory effect of NaB on the migration of SW620 cells was increasing. After the intervention of NaB, the migration ability of cells in each group showed a downward trend. At the 6th hour after scratch, there was no significant difference between the intervention groups and the control group ($P > 0.05$), but at the 24th and 28th hours after the scratch, there were significant differences between the intervention groups and the control group ($P < 0.05$). The results were shown in Figure 2.

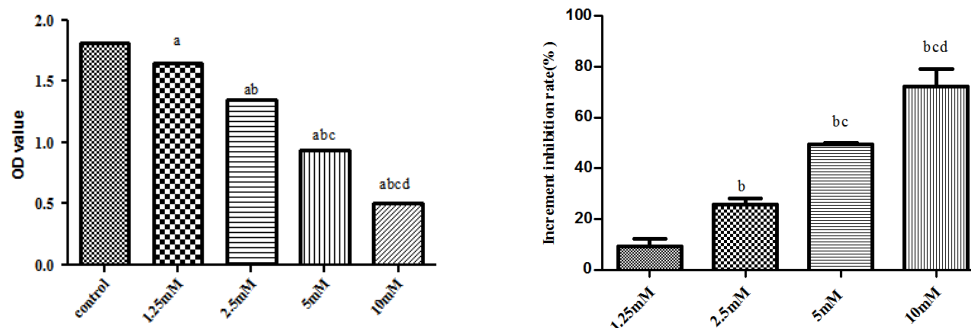


Figure 1. The effect of NaB at different concentrations on proliferation activity of SW620. Note: a: compared with control group $P < 0.05$; b: compared with 1.25mM group $p < 0.05$; c: compared with 2.5mM group $P < 0.05$; d: compared with 5mM group $P < 0.05$.

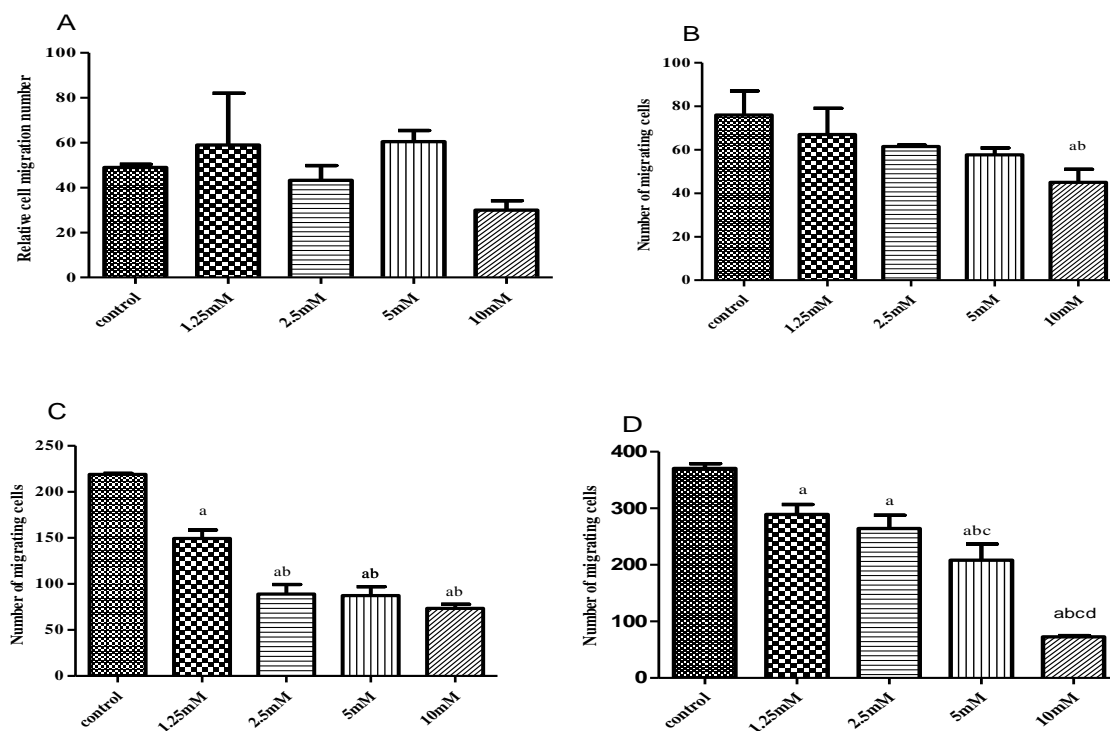


Figure 2. Migration of SW620 cells at different time points after sodium butyrate intervention. Note: A: Relative migration number of cells on both sides of scratch 6 hours after scratch; B: Relative migration number of cells on both sides of scratch 12 hours after scratch; C: Relative migration number of cells on both sides of scratch 24h after scratch; D: Relative migration number of cells on both sides of scratch 48 hours after scratch a: compared with control group $P < 0.05$; b: compared with 1.25mM group $P < 0.05$; c: compared with 2.5mM group $P < 0.05$; d: compared with 5mM group $P < 0.05$.

3.3. Different concentrations of NaB could restrain the migration ability of SW620 cells

The upper chamber of the transwell chamber was observed under an inverted microscope, and each of the chambers was randomly selected from five fields to observe the number of migrated cells on the chamber membrane. The final number of migration results is the average of the number of cells counted five times. Transwell chamber migration experiment showed that NaB could significantly reduce the migration of SW620 cells ($P < 0.05$). There were significant differences between 1.25mM intervention group and 5mM, 10mM groups, and between 5mM and 10mM groups ($P < 0.05$). The result was given in Figure 3.

3.4. Different concentrations of NaB could depress the invasiveness ability of SW620 cells

The upper chamber of the transwell chamber was stained and placed under an inverted microscope. Five visual fields were randomly selected, and the final results were counted as average values of five counts. Different concentrations of NaB could inhibit the invasiveness of SW620 cells in different degrees ($P < 0.05$, compared to control group). The number of

cell invasion in 1.25mM group was significantly different from that in 10mM group ($P < 0.05$). The higher concentration of NaB was applied, the more obvious inhibitory effect on the invasiveness of SW620 cells. The result was as showed in Figure 4.

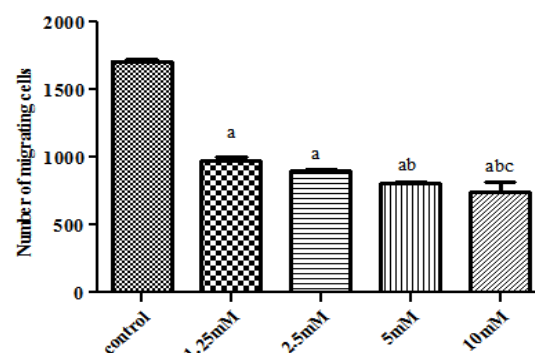


Figure 3. Migration effect of SW620 cells in different concentration of NaB. Note: a: compared with control group $P < 0.05$; b: compared with 1.25mM group $P < 0.05$; c: compared with 2.5mM group $P < 0.05$; d: compared with 5mM group $P < 0.05$.

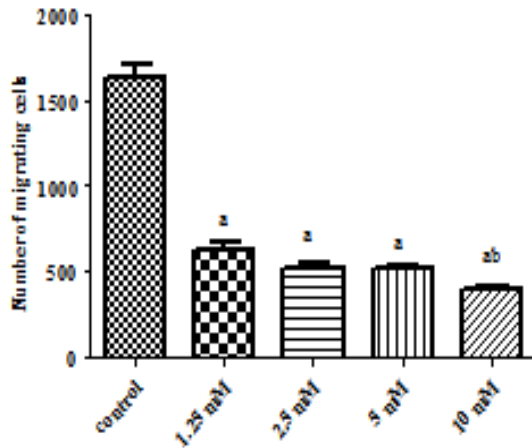


Figure 4. Invasion effect of SW620 cells in different concentration of NaB. Note: a: compared with control group $P < 0.05$; b: compared with 1.25mM group $P < 0.05$.

3.5. Different concentrations of NaB could reduce the adhesion ability of SW620 cells

The results of cell adhesion assay showed that the OD value of cells in the intervention group was significantly lower than that in the control group after the intervention of NaB ($P < 0.05$). The adhesion ability of SW620 cells was significantly inhibited by different concentrations of NaB. The higher the concentration of NaB was used, the more obvious the inhibitory effect on the invasiveness of SW620 cells. The results were shown in Figure 5.

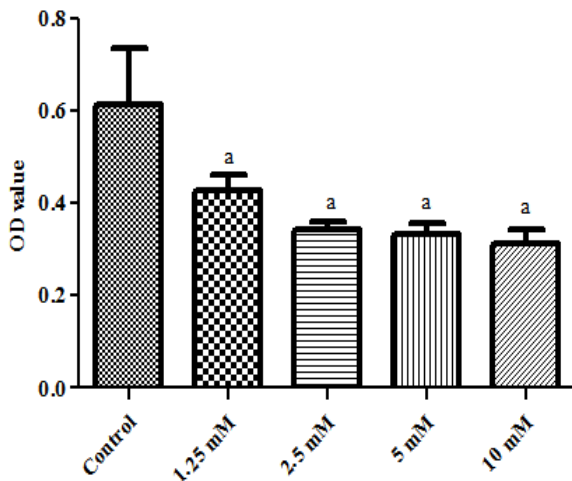


Figure 5. Adhesion effect of SW620 cells in different concentration of NaB. Note: a: compared with the control group, $P < 0.05$.

3.6. Different concentrations of NaB could inhibit the expression of TGF-β and Vimentin protein

and promote the expression of E-Cadherin in SW620 cells

The protein expressions of E-Cadherin, TGF-β and Vimentin in SW620 cells were measured by western-blot to further investigate the mechanism of NaB on CRC metastasis. Figure 6 showed the effects of different concentrations of NaB on the protein expressions of E-Cadherin, TGF-β and Vimentin. It was found that the expression of E-Cadherin was obviously decreased in the control group ($P < 0.05$), compared to other groups. Along with the increase of NaB concentrations, the protein of E-Cadherin expression was gradually increased. On the contrary, the protein expression of TGF-β was the highest in the control group ($P < 0.05$, compared to other groups), and gradually decreased along with increased of NaB concentration. Meanwhile, the protein expression of Vimentin was also the highest in the control group ($P < 0.05$, compared to other groups) and gradually decreased along with increase of NaB concentration. Concrete results were shown in Figure 7.

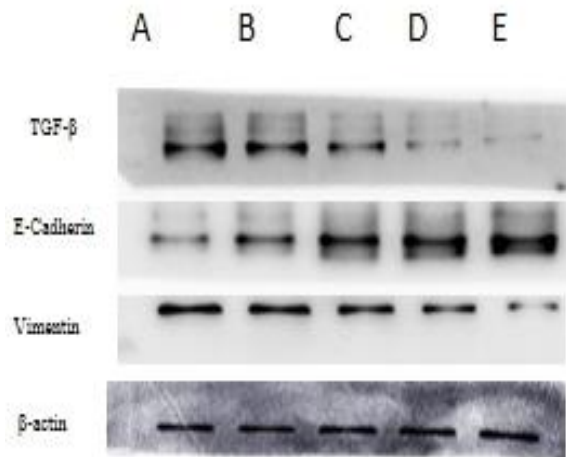


Figure 6. The effects of different concentrations of NaB on the expression of TGF-β, E-Cadherin and Vimentin in SW620 cells. Note: Group A: control group, group B: 1.25mM NaB, group C: 2.5mM NaB, group D: 5mM NaB, group E: 10mM NaB.

4. Discussion

The metastasis of CRC is a complicated, multifactorial and multistage process[15].The process of CRC metastasis can be roughly explained as follows: the primary tumor cells lose their polarity, the loss of intercellular adhesion factors leads to the enhancement of the movement and invasion of tumor cells (that is the EMT process), the basement membrane is degraded, and the tumor cells enter the circulatory system. The tumor cells reach the distant tissues with the circulatory system and are separated from the immune system to develop metastatic tumors[16,17].

EMT refers to the transformation of polar epithelial cells into stromal cells which move freely between cell matrix under specific physiological and pathological conditions. The concept of EMT was put forward by Greenburg et al in 1982[18]. They found that epithelial cells of embryonic and adult lenses cultured in

three-dimensional collagen gels could form pseudopodia and then transform into stromal like cells. At present, EMT is considered to be a key step to initiate tumor metastasis, which can be involved in the invasion and metastasis of many kinds of tumors.

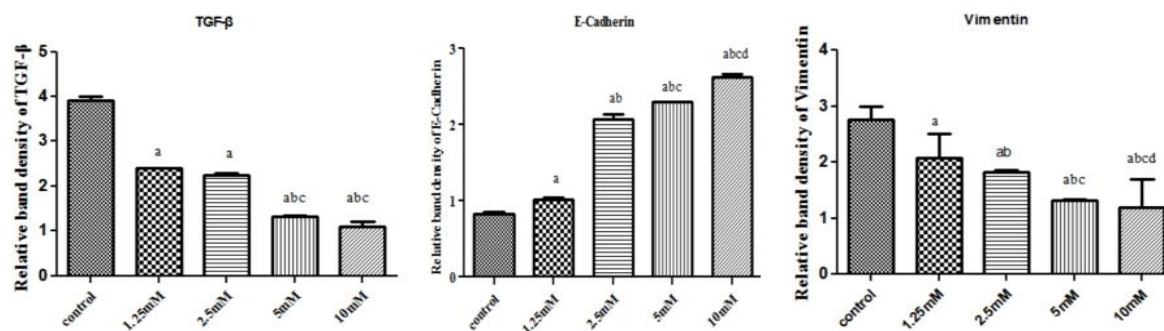


Figure 7. Effects of different concentrations of NaB on the expression of TGF-β, E-Cadherin and Vimentin protein in SW620 cells. Note: a: compared with control group $P < 0.05$; b: compared with 1.25mM group $P < 0.05$; c: compared with 2.5mM group $P < 0.05$; d: compared with 5mM group $P < 0.05$.

In order to study whether NaB inhibits the EMT process of CRC cells, we selected SW620 cells to observe the inhibitory effect of NaB on EMT process of CRC cells. As a common histone deacetylase (HDAC), NaB is capable of inhibiting histone deacetylation and enhancing the degree of histone acetylation, which changes chromatin from a dense repressor structure to a relaxed transcriptional activation structure, and facilitating the binding of transcription factors to DNA and activating related genes[19]. Our results showed that the proliferation, migration, invasion and adhesion of SW620 cells were significantly inhibited by NaB, which is consistent with the experimental results of Wang[20]. In order to further study the inhibitory effect of sodium butyrate on EMT in colorectal cancer cells, we studied several key proteins of EMT.

TGF-β, as one of the most common leading factors of EMT, can regulate the EMT process of cells through multiple signal pathways[21,22]. TGF-β activates many transcription factors through Smad signaling pathway, such as ZEB1, SIP1, Snail, Slug, Twist and so on. These nuclear transcription factors can induce down-regulation of tight junction proteins such as ZO-1 and Claudin-1, down-regulation of epithelial biomarkers such as E-Cadherin, and up-regulation of interstitial biomarkers such as Vimentin and N-Cadherin. Then the epithelium-derived tumor cells lose polarity, show fibroid phenotype, the ability of adhesion decreases, and the ability of cell migration and invasion increases. E-Cadherin is a kind of transmembrane glycoprotein of mediates cell adhesion, mainly expressed in epithelial cell membrane, includes N-terminal extracellular domain, highly hydrophobic

transmembrane region and C-terminal intracellular region. E-Cadherin forms a "zip-like" adhesion junction system mainly through the extracellular portion of β-catenin between cells. This system has long been recognized as an "infiltration inhibition system," and its reduced or missing expression can lead to reduced adhesion between cancer cells, prone to abscission and metastasis. Therefore, the decreased or missing expression of E-cadherin is considered to be a marker of EMT. Vimentin belongs to the intermediate fiber family and forms a cytoskeleton network with microtubules and filaments to maintain the integrity of cells. Vimentin is mainly distributed in mesenchymal tissues and cells. Vimentin expression was also found in malignant tumor cells, such as lung cancer, bladder cancer, cervical cancer, and abnormal expression of Vimentin was closely related to tumor cell differentiation, invasion and metastasis[23]. CY Ngan[24] found that the overexpression of Vimentin was positively correlated with the malignant degree of CRC. Therefore, Vimentin is often considered as a marker of EMT. Our study found that NaB could inhibit the EMT process of tumor cells by down-regulating the expression of TGF-β and Vimentin, and up-regulating the expression of E-Cadherin. The above results suggest that NaB can inhibit the metastasis of CRC directly or indirectly by regulating the key proteins related to EMT of tumor cells and inhibiting the EMT process of tumor cells.

In our experiments, we found that different concentration NaB could inhibit the proliferation, migration, invasion, adhesion and regulate EMT related process in SW620 cells. These results suggest that NaB can inhibit the invasion and metastasis of

CRC by inhibiting the EMT process only at the concentration under millimoles. The present study provide strong evidence for the inhibition of CRC invasion and metastasis by NaB.

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