c-MET Protects Breast Cancer Cells from Apoptosis Induced by Sodium Butyrate

Bo Sun1*, Rui Liu2, Zhong-Dang Xiao1, Xuan Zhu3*

1 State Key Laboratory of Bioelectronics, School of Biological Science and Medical Engineering, Southeast University, Nanjing, China, 2 Laboratory of Biophysics, School of Biological Sciences, Seoul National University, Seoul, South Korea, 3 School of Pharmaceutical Science, Xiamen University, Xiamen, China

Abstract

Sodium Butyrate (NaBu) is regarded as a potential reagent for cancer therapy. In this study, a specific breast cancer cell population that is resistant NaBu treatment was identified. These cells possess cancer stem cell characters, such as the capability of sphere formation in vitro and high tumor incidence rate (85%) in mouse model. Forty percent of the NaBu resistant cells express the cancer stem cells marker, the CD133, whereas only 10% intact cells present the CD133 antigen. Furthermore, the endogenous expressing c-MET contributes to the survival of cancer stem cell population from the treatment of NaBu. The CD133+ group also presents a higher level of c-MET. A combination treatment of MET siRNA and NaBu efficiently prohibited the breast cancer progression, and the incident rate of the tumor decrease to 18%. This study may help to develop a new and alternative strategy for breast cancer therapy.

Introduction

Sodium butyrate (NaBu) is the sodium salt of butyric acid produced by large intestinal micro flora. As a potent histone deacetylase (HDAC) inhibitor in vivo, NaBu has been reported to regulate a large number of genes in cultured mammalian cells [1]. It has been reported that the NaBu induce growth arrest, apoptosis or differentiation on different cancer cell lines, including breast cancer cell lines [2]. Since it exhibits cancer chemotherapeutic potential, NaBu has been considered as a potential regent for cancer therapy [3]. Recently, It was reported that NaBu induces breast cancer cell differentiation by regulating β-casein and N-myc downstream-regulated gene 1 (NDRG1) in breast cancer cells [4]. Caspase-10 also plays an important role in the induction of apoptosis by NaBu on breast cancer cells [5]. However, cancer is composed of heterogeneous population of cells and the function of NaBu on different population needs to be elucidated.

MET oncogene, encodes for the tyrosine kinase receptor for hepatocyte growth factor, has been shown to be over expressed in various type of tumor cells [6,7]. It contributes to the invasive growth of cancer cells through hepatocyte growth factor paracrine stimulation. Within the intracellular portion of MET, Tyr1234 and Tyr1235, mediates MET biological activity and the key tyrosine residues in the carboxy-terminal tail, Tyr1349 and Tyr1356 capable of recruiting downstream adapter proteins with Src homology-2 (SH2) domains [8]. Several oncogenic pathways are recruited by the engagement of MET, including Ras-Erk/ mitogen-activated protein kinase (MAPK) pathway, Rac1/Cdc42-PAK pathway and Gab1-phosphoinositide 3-kinase (PI3K)-Akt pathway [8,9,10]. Moreover, c-MET may work with WNT or NOTCH pathways for the self renewal of cancer cells or stem cells [11].

In breast tumor patient, the relationship between c-MET up-regulation and tumor progress has been demonstrated [12]. Studies revealed MET overexpression correlated with aggressive phenotype of different cancer, including breast cancer. Indeed, c-MET has been regarded as a novel target for therapeutic approaches because of the significant correlation between c-MET overexpression and a high risk of disease progression [13,14]. However, the interaction between c-MET and NaBu, the HDAC inhibitor is not clear. In the present study, we found that c-MET protects breast cancer cells from the apoptosis induced by NaBu. Moreover, we also demonstrated that a NaBu- resistant population expressed a high level of c-MET with cancer stem cells property. The result also indicated that, although regarded as a tumor suppressor, NaBu might be not sufficient to remove cancer stem cells and to prohibit the recurrence of breast cancer.

Results

Anti-tumor efficiency of NaBu was different in MDA-MB-231 cells and MCF-7 cells

In order to test the tumor suppression effect of NaBu in different types of breast cancer, two kinds of breast cancer cell lines, the estrogen receptor negative MDA-MB-231 and the estrogen receptor positive MCF-7, were rendered the treatment of different concentration of NaBu (1–5 Mm, data not shown) for 2 days. It was observed that the MDA-MB-231 cell line showed a better survival rate than the MCF cell line (Fig. 1A–D). Since the most significant different survival rate between the two cell lines presents under 4 mM Nabu treatment, we applied this concentration for studies thereafter. Nearly 30% of MDA-MB-231 cells remained viable with only 16% of MCF-7 cells survived under the same condition when treated with 4 mM Nabu, as assessed by...
MTT assay (Fig. 1E). The 4 mM Nabu treatment showed the most significant survival rate between the two cell lines, therefore, we applied this concentration for further studies. Since previous studies demonstrated that NaBu induced cell apoptosis in MDA-MB-231 and MCF-7 [15,16]. Herein, we investigated whether the different survival rate between these two cell lines is caused by their different response to the treatment of NaBu. Indeed, western blotting results showed higher expression level of Bad, Bax and Cytochrome C, but weaker expression level of Bcl-2 in MCF-7 cells than in MDA-MB-231 cells after NaBu treatment (Fig. 1F and Figure S1). These results suggested that the MDA-MB-231 cells were more resistant to apoptosis effect after NaBu treatment as compared to MCF-7 cells. By knocking down the expression of Bad or Bax, the apoptosis effect of NaBu was attenuated in MDA-MB-231 cells (Figure S2). These results suggested that the NaBu caused the MDA-MB-231 cells loss by apoptosis induction effect.

c-MET contributed to the survival of breast cancer cells after the treatment of NaBu

Studies have illustrated that MET, an oncogene that promotes the progression and invasion of cancer cells, contributes to the cell proliferation by minimizing the apoptosis [7]. We demonstrated that higher expression level of MET in MDA-MB-231 cells than in MCF-7 cells by RT-PCR assay, which was consistent with previously studies (Fig. 2A) [17].

To confirm that the survival rate differences between MCF-7 and MDA-MB-231 cells result from the difference of MET expression in the two types of cells, we knocked down expression of MET in MDA-MB-231 cells by transfection with c-MET siRNA. After the transduction of MET siRNA, the expression of MET was diminished (Fig. 2B), while after treatment with NaBu, siMET transfected MDA-MB-231 cells resulted in a significantly lower survival rate compared to the NaBu treated con siRNA transfected cells (control group) (Fig. 2C). It suggested that the survival rate of con siRNA treated cells and intact cells were similar (data not shown). On the other hand, after we enhanced the expression of MET in MCF-7 cells (Fig. 2D), the cell survival rate was increased from 12% to 26% after treated with NaBu (Fig. 2E). Thus the oncogene MET helps breast cancer line survival through the treatment of NaBu. Since MDA-MB-231 cells relatively express higher MET level, we focused our studies on MDA-MB-231 cells in the subsequent studies.

NaBu- resistant MDA-MB-231 cell showed cancer stem cells characteristics

Studies have shown that c-MET is closely related to the cancer stem cell phenotype and is associated with SDF-1-CXCR4 and LIF-R-LIF axes for the trafficking of normal and malignant stem cells [10]. We wondered if the NaBu- resistant population in MDA-MB-231 cells displayed a higher cancer stem cell capability. Firstly, we examined the NaBu- resistant population with an elevated expression of MET compared to the control group (Fig. 3A). This was consistent with the western blot results (Fig. 3B; we mixed up figure 3B previously and all of our available data show c-MET upregulation in NABU treated cells, shown as Figure S3). Since c-MET helps the NaBu-resistant population survival as we demonstrated above, therefore, it could be concluded that NaBu selected c-MET positive cells out and enriched them in the dish.

To determine the stem cell capability of NaBu-resistant MDA-MB-231 cells, sphere formation tests were performed. Firstly, we confirmed that the con siRNA treated cells have similar sphere formation ability with intact cells. The con siRNA treated or MET siRNA treated cells were applied in this experiment. NaBu-resistant breast cancer cells showed a significantly higher sphere formation ability than the con siRNA treated group (Fig. 3C). As expected, the sphere formation ability of NaBu resistant MET knock down group was dramatically decreased (Fig. 3C), comparing to NaBu-resistant con siRNA treated group. These results suggested that NaBu treatment enriched the c-MET positive cancer cell population which possesses the breast cancer stem cell capability.

Afterwards, We employed CD133 for the confirmation of breast cancer stem cells character of NaBu- resistant population, since...
Figure 2. c-MET helped the survival of breast cancer cells after the treatment of NaBu. RT-PCR result illustrated that the expression level of MET was higher in MDA-MB-231 cell than in MCF-7 cells (A). The expression level of MET in MDA-MB-231 cells were decreased by the treatment of MET siRNA (B). Silencing MET in MDA-MB-231 cells resulted in a poor survival rate (7%) when the cells were treated by NaBu (C). For MCF-7 cells, while the MET expression was increased obviously by induction of MET activator (D), the cell survival rate were increased significantly with the presence of NaBu in culturing medium (26%)(E). *P < 0.05.
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Figure 3. NaBu-resistant MDA-MB-231 cell showed cancer stem cells character. RT-PCR result indicate that the NaBu- resistant cell population displays a higher expression level of MET (A). This was consistent with the western blot results (B). After seeding 10^5 cells in 100 mm peridish, NaBu- resistant cells showed the strongest sphere formation capability (2.7 × 10^4/dish) when compared with control siRNA treated group(1.8 × 10^4/dish)and MET siRNA treated group(0.8 × 10^4/dish)(C). A higher CD133 expression level by western blot in the NaBu- resistant group was observed(D). More than 40% NaBu- resistant cells were positive for CD133 expression, however, within the control siRNA treated cells, only around 10% cell were positive for CD133(E). The CD133^+ cells expressed higher level of c-MET compare to CD133^− cells(F). After treated with NaBu, cell viability of CD133^+ cells are significantly higher than that of CD133^− cells number . Intact MDA-MB-231 cells are used as control without the treatment of NaBu (G). *P < 0.05.
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CD133 is regarded as a marker for breast cancer stem cells [18]. We obtained a higher CD133 expression level by western blot in the NaBu-resistant cell initiated sphere (Fig. 3D). Correspondingly, we found that in the NaBu-resistant cell initiated spheres, 40% cells expressed CD133 positively. However, only nearly 10% cells in control cell initiated spheres presenting CD133 antigen (Fig. 3E). To elucidate whether c-MET expressed higher in CD133+ population, we tried to separate CD133+ and CD133- cells in intact MDA-MB-231 cell initiated spheres by flow cytometry. The expression of c-MET in these two groups was checked by western blot. As we expected, the CD133-positive cells expressed higher level of c-MET, compared to CD133-negative cells (Figure 3F). To testify whether the CD133-positive and the CD133-negative cells respond differently to NaBu, We separated CD133+ and CD133- cells from intact MDA-MB-231 initiated sphere. The same number (10^4/ml) of the two fraction cells were cultured with 4mM NaBu in 100 mm dish. Two days later, NaBu was removed by changing the medium. After one day, MTT assay was performed. After treated with NaBu, cell viability of CD133+ cells was significantly higher than that of CD133- cells number. Intact MDA-MB-231 cells are used as control without the treatment of NaBu (Fig. 3G).

NaBu-resistant MDA-MB-231 cell possessed an increasing tumor initiation ability in vivo

Since the NaBu-resistant group displayed cancer stem cells property as described above, we subsequently testified the tumor initiation ability in an animal model. The same number of NaBu-resistant cells and intact MDA-MB-231 cell was transplanted into mouse mammary fat pat. Tumors became palpable as early as on the 5th day in the mice transplanted with NaBu-resistant cells. However, in the control group, tumors became palpable on the 10th day post transplant. By the day 28th, tumor volume was significantly different between the two groups (Fig. 4A). Compare with NaBu treated intact cells, or with MET knock down cells, tumor volume was significantly smaller in NaBu treated MET knock down cell transplanted group (Fig. 4A). Meanwhile, we tried to clarify the tumorigenic effect of MET in the same animal model. MET knocked down MDA-MB-231 cells were generated before treated with 4mM NaBu for 2days. The NaBu-resistant MET knock down cells and con siRNA transfect cells were also transplanted into the mouse. The tumor incidence in intact MDA-MB-231 group and con siRNA group was comparable (Fig. 4B). On the 30th day, the tumor incidence in NaBu-resistant MET knock down group was decreased to 18% as compared to con siRNA transfected cells (Fig. 4B). Again, the MET knock down resulted in a comparable tumor incidence rate with con siRNA treated control group (Fig. 4B). These results demonstrated that NaBu-resistant population of MAD-MB-231 cell has high tumor initiation ability, while down regulation of MET gene results in decreasing its tumorigenicity efficiently.

Discussion

It has been well elaborated that NaBu exerts its tumor suppression effect on several type of cancer, such as, colon cancer, lung cancer and breast cancer [15,19,20,21]. Therefore, NaBu has been regarded as a potential cancer therapy regimen if one despite the short life-time of NaBu in the body [20]. In the present study, we found that, although NaBu induces majority cell death in the breast cancer cells, the minority cells that share the breast cancer stem cell characteristics may survive through NaBu selection. Those cells display higher ability of sphere formation in vitro as well as higher tumor incidence in animal model. Our studies suggested that the potential anti-tumor regent NaBu treatment may not be capable of removing breast cancer efficiently, since the seed of breast cancer, breast cancer stem cells, may resist from the treatment.

Our study clarified that the oncogene MET contributed to the NaBu-resistant effect of breast cancer cell. MET expressing breast cancer cell population display cancer stem cell characteristics, and studies illustrated that c-MET is closely related with highly aggressive cancer cell type. c-MET attenuates the apoptosis effect induced by NaBu in breast cancer cells. Our results demonstrated that when cancer cells after treated by the combination of NaBu and MET siRNA, decreased tumor incidence dramatically in the mouse model. However, in this study, a cell line was used as a simplified model to testify the effect of NaBu and c-MET on breast cancer. Further studies are needed to examine the reaction of breast cancer cells, especially cancer stem cells, to NaBu and c-MET in the patient, since cancer stem cells are resident in and dynamically regulated by its specific micro-environment. Based on this result and other studies, the MET expression level is different between the two cancer cell lines, MCF-7 and MDA-MB-231.

Figure 4. NaBu-resistant MDA-MB-231 cells displayed a high tumor initiation ability in vivo. Intact MDA-MB-231 and NaBu-resistant MDA-MB-231 cells were transplanted into NOD/SCID mouse. By the day of 28th, the tumor volume were significantly different. Moreover, tumor became palpable as early as on the 5th days in the NaBu-resistant MDA-MB-231 cell transplanted group whereas on the 10th day in the control group(A). Compared with NaBu treated intact cells, or with MET knock down cells, tumor volume was significantly smaller in NaBu treated MET knock down cell transplanted group (A). The tumor incidence data were collected on the 30th day after transplanting cancer cells. The NaBu-resistant MDA-MB-231 cell transplanted group resuted in a similar tumor incidence rate as the intact MDA-MB-231 group(85%). Down regulation of MET with a treatment of NaBu leads to decrease the tumor incidence rate effectively(18%), although con siRNA teated group resulted in a similar tumor incidence rate with the intact cancer cell group(83%). *P<0.05. doi:10.1371/journal.pone.0030143.g004
of 2 mg/ml) in 16 was determined using an Ultra-microplate reader (ELx 808; Bio-

each well and incubated 1 hr on an orbital shaker. The A570 nm
treating breast cancer cells.

A combination treatment of MET siRNA and NaBu administra-
cancer stem cell population survived after the treatment of NaBu.

whether another protective pathway exist to support cancer cells
resisting the treatment of NaBu.

For the colony formation assay, studies were preformed
for tumor-sphere counting after 9 days. Colonies of at least 60
were chosen to verify the sparseness of each spherical culture, and only wells
seeded, each well was checked under the microscope to

two days in fresh medium without NaBu. To testify the effect of c-

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Recombinant retroviruses were constructed by subcloning full-
length human cDNA of Met into the retroviral expression vector
pLNCX2 (Clontech, CA), which carries a neomycin phospho-
transferase cassette. A retroviral expression vector carrying the
cDNA of human placenta alkaline phosphatase (pLAPSN) served
as a control. Experiments were conducted according to the
manufacturers’ instructions and previous report [23].

After determining the cell viability by trypan blue exclusion test, 2 × 106
were injected into mammary fat pad in a 100 uL volume of

Animal studies

All mouse studies were performed in accordance with protocols
approved by the Animal Care and Use Committee at Southeast
University. SCID mice were housed under pathogen-free conditions and were
given autoclaved food and water. MDA-MB-231 cells were exposed to NaBu for 2
days. The intact MDA-MB-231 cells were used as control group.

Western blot analysis

Protein was extracted from the cells after culturing in the
indicated conditions. After measuring the protein concentrations
of homogenized lysates, 10 µg of protein extracted from cancer
was separated by 10% SDS-PAGE and transblotted onto a
polyvinylidene fluoride (PVDF) membrane. After blocking in a
powdered nonfat milk solution (5% in PBS) with 0.05% Tween-20,
the blot was incubated with a polyclonal rabbit anti-human Bel-2-
associated death promoter (BAD) antibody (Cell Signaling,
Danvers, MA), a rabbit anti-human BAX antibody (Cell
Signaling), a rabbit anti-human Bel-2 antibody (Cell Signaling), a rabbit
anti-human Cytochrome C antibody (Cell Signaling), a rabbit
anti-human CD133 antibody (Cell Signaling) and a rabbit
anti-human beta-actin antibody (Cell Signaling) at 1:1000
for 10 min, and DMSO (120 µL, Sigma) was added to
each dish, and the cells were



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sterile phosphate-buffered saline. SCID mice were used as 6 weeks of age and 10 mice for each group. Tumors were measured using precision calipers twice weekly. Tumor volume was calculated at:

\[ \text{volume} = \left( \text{length} \times \text{width} \right)^2 / 2. \]

**Flow cytometry**

After 3 washes with PBS, cells that were treated with NaBu and intact cells were detached with trypsin (Gibco) for 10 min at 37 °C. For cell surface antigen phenotyping, floating and detached spindle-shaped cells were stained with fluorochrome antibody CD133 (Becton–Dickinson, San Jose, CA, USA). Analyses were performed with FACS Calibur (Becton–Dickinson, NY, USA). CD133 positive cells were sorted by DAKO cytometry (DAKO, Carpinteria, CA).

**Statistical analyses**

Data are presented as means ± standard error (SD) in quantitative experiments. The differences between groups were analyzed using the unpaired Student’s t-test. P values <0.05 were considered significant.

**Supporting Information**

**Figure S1** The expression of Bad in MDA-MB-231 and MCF-7 cells (A). The expression in the MDA-MB-231 cells compared to the MCF-7 cells, triplicate studies were performed. The expression of BAD in MDA-MB-231 and MCF-7 were detected by western blot (A). The results were quantitated and presented a significant difference in Bad expression in between MDA-MB-231 and MCF-7 cells (B).

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