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# Novel Protein pp3501 Mediates the Inhibitory Effect of Sodium Butyrate on SH-SY5Y Cell Proliferation

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## ABSTRACT

Sodium butyrate, a new potential therapeutic drug, improves the efficacy of chemo- and immunotherapy of cancer under unknown mechanisms. A novel gene pp3501 is significantly induced in SH-SY5Y neuroblastoma cells upon sodium butyrate treatment. Therefore, this study has cloned pp3501 cDNA by RT-PCR and generated its recombinant fusion protein and anti-serum subsequently. The pp3501 protein localized mainly in the nucleus, as detected by immunocytochemistry and the expression of pp3501-EGFP fusion protein. pp3501 inhibited the proliferation of SH-SY5Y cells, arrested the cell cycle at G1 phase, and sensitized the SH-SY5Y cells to sodium butyrate treatment. These results provide a new mechanism of sodium butyrate inhibiting cancer cell proliferation as well as a new avenue for the future research on the functions of pp3501. J. Cell. Biochem. 113: 2696–2703, 2012. 

**KEY WORDS:** LOCALIZATION; pp3501; RECOMBINANT; SH-SY5Y CELL; SODIUM BUTYRATE

Earlier reports [Lane and Chabner, 2009; Ma et al., 2009] have shown that histone deacetylase inhibitors (HDACIs) regulate the expression of several sets of proteins that are involved in the regulation of cell cycle, proliferation, differentiation, and apoptosis, which support that HDACIs are promising new class of antineoplastic agents for tumor treatment.

Sodium butyrate, one of the HDACIs, has been shown to have significant anti-tumor activity in various cancer cells. Studies by Bandyopadhyay et al. [2004] have demonstrated that sodium butyrate induces apoptosis in metastatic melanoma cells by increasing acetylation of p53 and upregulating Bax. Earlier reports by Louis et al. [2004] have also shown that sodium butyrate increases Bax expression and downregulates Bcl-2 level in breast cancer cell. Mohana et al. [2007] have demonstrated that sodium butyrate arrests cell cycle at the G0/G1 phase and alters the expression of Bax, Bak, and Bcl-2. The results from Hara et al. [2000] have suggested that butyrate induces apoptosis through upregulation of proapoptotic Bak and downregulation of antiapoptotic Bcl-xL, which were also observed by Ruemmele et al. [2003]. Therefore, these results indicate that sodium butyrate-induced apoptosis in some cancer cells is mediated by the regulation of bcl-2 family expression.

Cho et al. [2006] have shown that sodium butyrate induces caspase-3 and poly-ADP ribose polymerase cleavage and up-regulation of bax, implying that mitochondrial damage is involved in sodium butyrate-induced apoptosis in prostate cancer cells.

Pajak et al. [2007] have reviewed relevant previous works and summarized that the molecular events of sodium butyrate-induced apoptosis are mediated by both intrinsic and extrinsic apoptotic pathways.

Recently, sensitization of cancer cells to the cytotoxic effects of radiation and anticancer drugs by sodium butyrate becomes the focus of attention. For example, Singh et al. [2005] have shown that the combination of Artemisinin and sodium butyrate exerts synergistic cytotoxicity on human cancer cells. Other studies by Pajak et al. [2009] have demonstrated that the combination of TNF-alpha and sodium butyrate treatment induces cleavage of Bid and activation of caspase-9 in human colon adenocarcinoma. Studies by Ogawa et al. [2004] have shown that sodium butyrate enhances the Fas-mediated apoptosis in human hepatoma cells.

Conflicts of interest: None.

Yajun Wang and Chao Ma contributed equally to this work.

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Although sodium butyrate forms a good candidate as the new therapeutic strategy to improve the chemo- and immunotherapy of cancer, the underlying molecular mechanisms remain elusive.

Sodium butyrate modulates gene expressions in vitro. A hypothetical protein pp3501 (GeneBank Accession: AF218008.1, GI: 10441945) identified by Wan et al. [2004], has been specifically induced in sodium butyrate-treated Raji and SH-SY5Y cells. This study aims to clone the pp3501 cDNA and investigate the effect of pp3501 on the proliferation of SH-SY5Y cells.

## MATERIALS AND METHODS

### CELL CULTURE

Neuroblastoma cell line SH-SY5Y was cultured in the Dulbecco's Modified Eagle Medium (Gibco BRL, Grand Island, NY) containing penicillin (100 μg/ml) and streptomycin (100 μg/ml) and supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% $CO_2$.

### AGENTS AND CHEMICALS

Sodium butyrate, 3-(4,5-Dimethyl-thiazol-2-yl)2,5-diphenyl-tetrazolium bromide (MTT), Freund's adjuvant, Hoechst33258, RNase, and DNase I were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibodies against GFP, GAPDH, and LaminB1, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA).

### CLONING OF pp3501 CDNA BY RT-PCR

SH-SY5Y cells were treated with 3 mmol/L sodium butyrate at 37°C in a humidified atmosphere with 5% $CO_2$ for 48 h. Total RNAs were isolated from cultured cells using Trizol reagent (Invitrogen, Carlsbad). Before cDNA was synthesized, total RNA was digested by DNaseI. cDNA was synthesized using RT-PCR kit (Dingguo, Beijing, China) according to the manufacturer's instruction. Sequences of the primers for the pp3501 gene were5′-CTGGGCTCGGGTGATCCTCCT-3′ and 5′- GGGTCTTGCACGTGTCACTCGT-3′. PCR amplification was performed under the following conditions: 5 min incubation at 94°C, 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, ended by 3 min of extension at 72°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis, purified, and sequenced.

### PROKARYOTIC EXPRESSION AND PURIFICATION OF RECOMBINANT pp3501 PROTEIN

The ORF of pp3501 was cloned into Pet-32a prokaryotic expression vector, which was then named as Pet-32a-pp3501. The recombinant plasmid was transformed into *E. coli* BL21 (DE3). Positive *E. coli* BL21 (DE3) clones were selected, inoculated in LB medium containing 100 μg/ml ampicillin sodium, and cultured at 37°C overnight in a shaker; the bacteria were then added into fresh LB medium containing 100 μg/ml ampicillin, and cultured at 37°C for 3 h. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added into the medium subsequently to a final concentration of 1 mmol/L, and the bacteria were induced for another 5 h. Bacteria were harvested by centrifugation at 10,000 g at 4°C for 1 min. His-tagged recombinant pp3501 fusion protein was purified with Ni-IDA affinity chromatography resin (Novagen, Madison, WI). The product was analyzed by SDS–PAGE using 10 or 12% gel.

### PREPARATION OF ANTI-pp3501 POLYCLONAL ANTIBODY

About 1 mg of purified recombinant pp3501 protein was mixed thoroughly with 1 ml of complete Freund's adjuvant. The mixture was then injected into New Zealand white rabbits, which were injected every 7 days to enhance the immunity. The serum of rabbit was collected to determine the titer of antibody. Purified protein was diluted to 5 μg/ml, coated onto 96-well, and incubated at 4°C overnight. The coated plate was washed three times with PBST buffer in the next day and blocked with 5 mg/ml BSA at 37°C for 1 h. The plate was subsequently washed three times with PBS buffer. Serially diluted anti-serum was added into the pp3501 coated wells. After 1 h incubation at 37°C, the plate was washed three times in PBST buffer and further incubated at 37°C for 1 h with HRP-conjugated goat anti-rabbit secondary antibody (1:4,000 dilutions). After incubation, the plate was washed three times in PBST buffer, and substrate solution was added and incubated for 20 min at room temperature in the dark. Enzymatic reaction was terminated with 2 mol/L sulfuric acid, and the absorbance of each well was detected at wavelength of 492 nm with Varioskan Flash Reader (Thermo scientific).

### REAL-TIME PCR

SH-SY5Y cells were treated with 3 mmol/L sodium butyrate at 37°C in a humidified atmosphere with 5% $CO_2$ for 48 h. Total RNAs were isolated using Trizol reagent and digested by DNase I. Quantitative comparison of pp3501 mRNA levels was carried out using real-time PCR technology with beta-actin as the endogenous control. Primer sequences for the pp3501 gene were5′- CAGATGCAGCTAAGAT-GAGGTCAC-3′ and 5′-AATGTTCGCCCTCTGAAATCC -3′, and the beta-actin primers were 5′-TCCTCCTGAGCGCAAGTACTC-3′ and 5′-CTGCTTGCTGATCCACATCTG-3′. The amplification reactions were carried out according to the instruction of one step SYBR® prime Script® RT-PCR II kit (Perfect Real-time PCR, TAKARA Biotechnology CO., LTD, Dalian, China). Final concentration of pp3501-specific primers was 400 nM and the amount of total RNA template was 100 ng. Amplification was performed with an ABI7300 real-time PCR thermocycler (Applied Biosystems) initially at 42°C for 5 min. The denaturation at 95°C was done for 10 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. The differential expression of pp3501 mRNA was analyzed with ABI PRISM Sequence Detection software.

### SUBCELLULAR LOCALIZATION OF pp3501 PROTEIN IN SH-SY5Y CELLS

SH-SY5Y cells were treated with 3 mmol/L sodium butyrate at 37°C in a humidified atmosphere with 5% $CO_2$ for 48 h. After three times washing with PBS, cells were fixed with ethanol overnight. Subsequently, cells were blocked with PBS containing 10% BSA after five times washing with PBS. Anti-pp3501 rabbit serum was added into the wells and incubated at 37°C for 1 h. Cells were washed three times in PBS and further incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:50 dilution) at 37°C for 40 min. In addition, pEGFPC1-pp3501 was transfected into SH-SY5Y cells to

confirm the localization of pp3501. Cells were stained with Hoechst 33258 and observed under Eclipse ET2000-E fluorescent microscope equipped with an automatic photomicrograph system (Nikon, Japan). Nuclear and cytosolic proteins were fractionated using Nuclear and Cytoplasmic Protein Extraction Kit from Beyotime Institute of Biotechnology (Hai Men city, China) according to manufacturer's instructions; western blot analysis was carried out using anti-GFP antibody and anti-pp3501 homemade antibody respectively. SH-SY5Y cells were treated with 3 mmol/L sodium butyrate for 10 days to observe the morphological changes.

### CELL TRANSFECTION

SH-SY5Y cells were transfected with different vectors using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. Stable cell lines were screened against 600 μg/ml G418 (Invitrogen). G418 concentration was reduced to 300 μg/ml after 3 weeks of selection. Stably transfected cells were treated with sodium butyrate, and the cell viability was determined by MTT assay [Zhang et al., 2007] and clone formation assay [Wenqi et al., 2009]. Cell cycle was then analyzed by flow cytometry (EPICS XL, Coulter, Fullerton, CA) [Zhang et al., 2007].

### siRNA KNOCK-DOWN OF pp3501

Twenty-four hours prior to the transfection, cells were seeded in 6-well plates containing DMEM medium without antibiotics. Cells were transfected with 100 pmol siRNAs (Sense: 5′ CCGCAGACA-CAUUUGUUCU dTdT 3′, Antisense: 3′ dTdT GGCGUCUGUGUAAA-CAAGA 5′) and control siRNA (sc-37007, were purchased from Santa Cruz Biotechnology) respectively using lipofectamine 2000 and incubated for 24 h. The cells were then treated with 3 mmol/L sodium butyrate for another 48 h; the cell viability was determined by MTT assay [Zhang et al., 2007]. Total proteins were isolated for western blot analysis [Liu et al., 2010].

### STATISTICAL ANALYSIS

The data were represented as mean ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) with LSD test. All differences with $P < 0.05$ were considered as significant.

## RESULTS

### IDENTIFICATION OF PET-32A-pp3501 EXPRESSION PLASMID

The 432 bp cDNA fragment of pp3501 was cloned by RT-PCR (Fig. 1A) and confirmed through sequencing. The pp3501 cDNA was then sub-cloned into various plasmids including Pet-32a, pEGFPC1, pEGFPN1, and pIRSE2-EGFP for further experiments.

### EXPRESSION OF pp3501 PROTEIN IN *E. COLI* BL21 (DE3)

Recombinant pp3501 protein was produced in *E. coli* BL21 (DE3) by IPTG induction and was detected with 12 or 10% SDS–PAGE as a 34 kD protein (Fig. 1B). Although the predicted size of pp3501 is 16 kDa, Pet-32a vector has a his-tag about 18 kDa, so the final size of recombinant pp3501 is about 34 kDa. The his-tagged recombinant pp3501 protein was purified conveniently with Ni-IDA His Bind affinity chromatography column.

### DETECTING THE TITER OF ANTI-pp3501 SERUM

Rabbit-anti-pp3501 serum was prepared by immunizing rabbits with purified recombined pp3501 protein. The titer of anti-pp3501 serum was determined as 1:12,800 by indirect ELISA analysis (Fig. 1C).

### EFFECTS OF SODIUM BUTYRATE ON pp3501-EXPRESSING SH-SY5Y CELLS

The morphology of SH-SY5Y cells showed protrusions (Fig. 1D), and the cell proliferation was suppressed (Fig. 1E) in the presence of sodium butyrate. Furthermore, SH-SY5Y cells grew long axis as neurons when sodium butyrate treated them for 10 days (Fig. 1D). Both mRNA (Fig. 1F) and protein levels of pp3501 (Fig. 1G) were upregulated after 48 h exposure to 3 mmol/L sodium butyrate.

### SUBCELLULAR LOCALIZATION OF pp3501 PROTEIN IN SH-SY5Y CELLS

As shown in Figure 1H, pp3501 proteins were localized in the nuclei of SH-SY5Y cells, which showed punctuated green fluorescent. To further confirm the nuclear localization of pp3501 protein, we have generated constructs that link EGFP (enhanced green fluorescent protein) to C-terminus of pp3501 to test its subcellular localizations in SH-SY5Y cells. Most of the EGFP-pp3501 fluorescence appeared in the nuclei (Fig. 1I), which confirmed the immunofluorescence staining results shown in Figure 1H. In addition, anti-GFP antibody and anti-pp3501 homemade antibody recognize EGFP-pp3501 in the same band on western blot, indicating that pp3501 was recognized by homemade antibody too (Fig. 1J). Similar results were shown by western blot that most of EGFP-pp3501 was in the nuclear fraction (Fig. 1K).

### pp3501 SUPPRESSES THE PROLIFERATION OF SH-SY5Y CELLS AND AFFECTS CELL CYCLE

pp3501 expressed in cells transfected with pIRSE2-pp3501-EGFP (Fig. 2A). Cells were cultured for 4 days continuously, and their viabilities were determined with MTT assay. As shown in Figure 2B, the proliferation of pIRSE2-pp3501-EGFP transfected cells was significantly slower than that of pIRSE2-EGFP transfected cells and non-transfected cells.

Colony-forming assay has demonstrated that pp3501-transfected cells lost the colony-forming capacity compared with the vector control; the colonies formed in pIRES2-pp3501-EGFP and pIRES2-EGFP groups were 26.8 ± 5.6 and 98.7 ± 15 respectively ($P < 0.05$, Fig. 2C). Based on these observations, we concluded that the expression of pp3501 suppressed the colony-forming capacity of SH-SY5Y cells.

After cells were cultured for two days normally, cell cycle analysis was performed and showed that the number of cells in G1 phase has increased and the number of cells in S phases has decreased in pIRSE2-pp3501-EGFP transfected SH-SY5Y cells, indicating that expression of pp3501 has arrested the cell cycle of SH-SY5Y cells at G1 phase (Fig. 2D,E). However, cell cycle analysis has not shown the peak of subG1, one of the apoptotic markers.

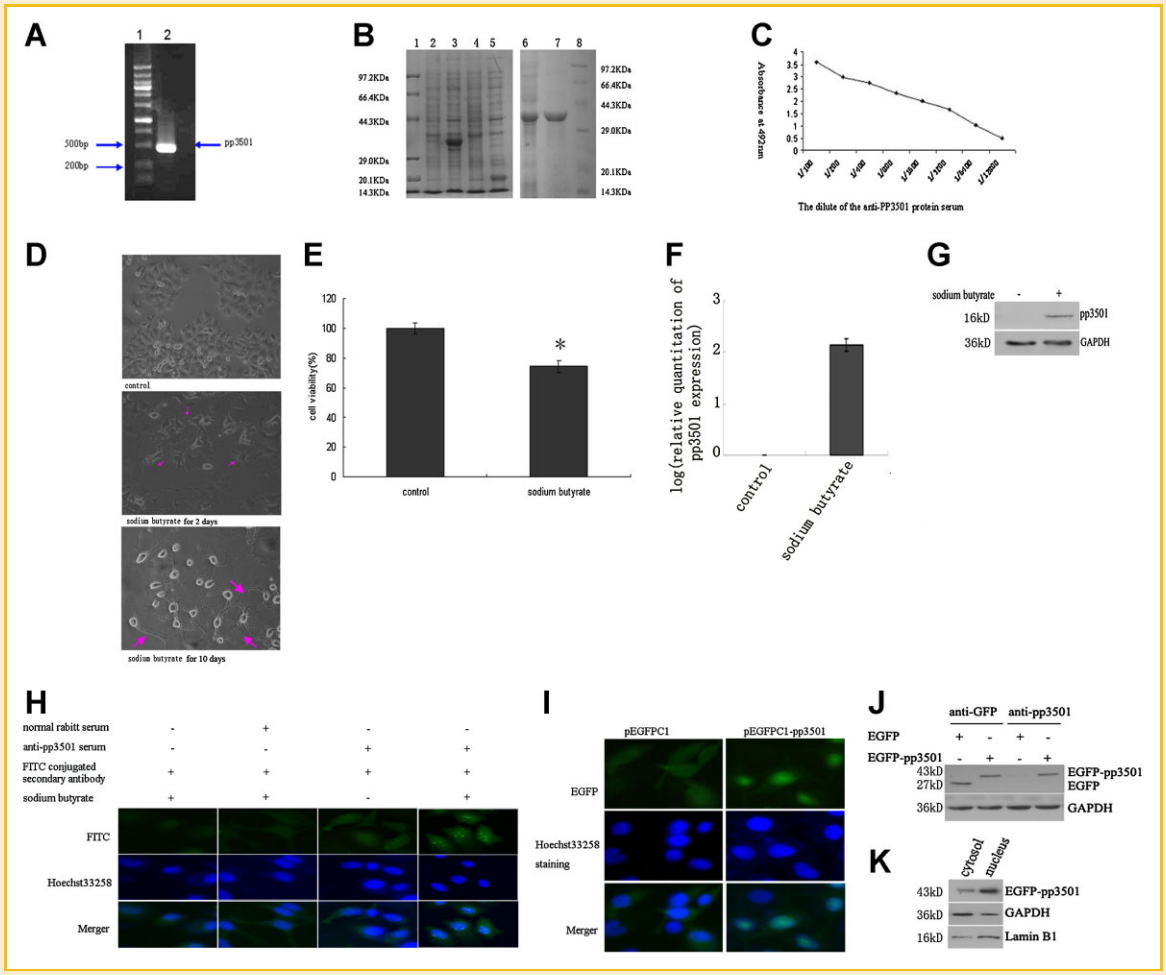


Fig. 1. Preparation of anti-pp3501 polyclonal antibody and subcellular localization of pp3501 protein in SH-SY 5Y cells. A: The SH-SY5Y cells were treated with 3 mmol/L sodium butyrate for 48 h. Total RNA was isolated and amplification of pp3501 cDNA was performed. The products were analyzed by 1.5% agarose gel electrophoresis.1, TaKaRa wide range maker; 2, pp3501 cDNA. B: The recombinant His-pp3501 protein was analyzed with 12 or 10% SDS–PAGE electrophoresis.1, Marker; 2, E. coli lysate transformed with Pet-32a-pp3501 vector, non-IPTG induced; 3, E. coli lysate transformed with Pet-32a-pp3501 vector, IPTG induced; 4, E. coli lysate transformed with control vector, non-IPTG induced; 5, E. coli lysate transformed with control vector, IPTG induced; 6, E. coli lysate; 7, purified recombinant pp3501 protein; and 8, marker. C: The curve of anti-PP3501 serum titer. Purified protein was diluted with coating solution. Serially diluted anti-serum was added into the pp3501 coated wells. The titer of anti-PP3501 serum was detected with enzymatic reaction assay with Varioskan Flash Reader. D: SH-SY5Y cells were treated with 3 mmol/L sodium butyrate for 48 h and 10 days (×200). Arrows indicate the protrusions of SH-SY5Y cells in the presence of sodium butyrate. E: The proliferation of SH-SY5Y cells was suppressed by 3 mmol/L sodium butyrate ($n = 4$; $^*P < 0.05$). F: Amplification of pp3501 mRNA with real-time PCR. G: pp3501 expression detected by western blot. H: Subcellular localization of pp3501 protein using homemade antibody. Nuclear staining by hoechst33258. pp3501 proteins are localized in the nucleus of SH-SY 5Y cells (×400). I: SH-SY5Y cells were transfected with pEGFPC1-pp3501, and nuclei were stained by hoechst33258. Results indicated pp3501 proteins are localized in the nucleus of SH-SY 5Y cells (×400). J: After transfection of SH-SY 5Y cells with different plasmids, proteins were isolated for western blot analysis with anti-GFP antibody and anti-pp3501 antibody. K: After transfection with pEGFPC1-pp3501, nuclear extract and cytoplasmic protein was isolated for western blot analysis with anti-GFP antibody. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

### pp3501 SENSITIZES SH-SY5Y CELLS TO SODIUM BUTYRATE-TREATMENT

Sodium butyrate exerted its anti-proliferative effect on SH-SY5Y cells in a dose- (Fig. 3A) and time-dependent (Fig. 3B) manner. The IC50 values of sodium butyrate against SH-SY5Y cells transfected with pIRSE2-EGFP and pIRSE2-pp3501-EGFP vectors were $7.0 \pm 0.46$ and $4.3 \pm 0.28$ mmol/L, respectively, while the IC50 value of sodium butyrate against untransfected SH-SY5Y cells was$7.1 \pm 0.32$ mmol/L. Although sodium butyrate induced cell expression of endogenous pp3501, the expression of exogenous pp3501 was significantly higher than endogenous pp3501. These data indicated that expression of exogenous pp3501 enhanced the anti-proliferative effect of sodium butyrate on SH-SY5Y cells ($P < 0.05$, vs. pIRSE2-EGFP control).

### pp3501 siRNA ALLEVIATES SH-SY5Y CELLS TO SODIUM BUTYRATE-TREATMENT

We tested the involvement of pp3501 in sensitizing SH-SY5Y cells to sodium butyrate-treatment by loss-of-function assay. The results of western blot analysis showed that the pp3501 expression was knocked down with siRNA (Fig. 4A). Simultaneously, the results of MTT assay clearly demonstrated that downregulation of pp3501

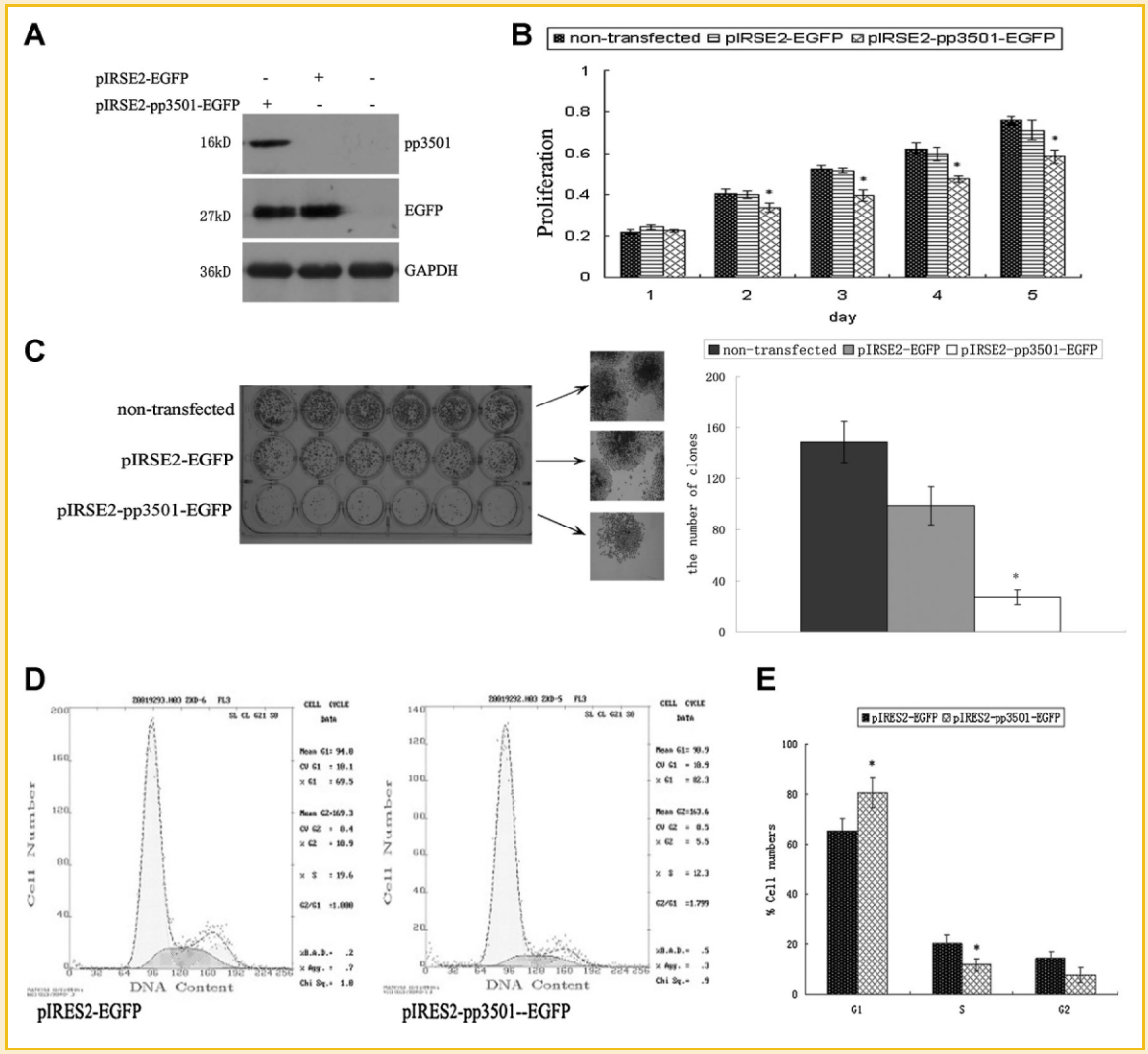


Fig. 2. Effect of pp3501 on SH-SY5Y cells. SH-SY5Y cells were transfected with pIRSE2-pp3501-EGFP or pIRSE2-EGFP vectors and screened for stable cell line with G418. Cell viabilities and colony formation ability have been determined. A: The expression level of pp3501 in transfected cells. B: The bar chart of proliferation of different group SH-SY5Y cells (mean ± SD; n = 6; $^{*}P < 0.05$, vs. pIRES2-EGFP-transfected group). C: The colony formation ability reduced by pp3501. SH-SY5Y cells, transfected with pIRSE2-pp3501-EGFP or pIRSE2-EGFP vectors, were cultured for 14 days. Cells were stained by Trypan blue, and the clones were counted under an invert microscope. The data are represented as mean ± SD (n = 6; $^{*}P < 0.05$ vs. pIRES2-EGFP-transfected group). D: Analysis of the effect of pp3501 on cell cycle by flow cytometry. E: The bar chart of cell cycle. The data are represented as mean ± SD (n = 3; $^{*}P < 0.05$ vs. pIRES2-EGFP-transfected group).

alleviated the sodium butyrate-induced inhibitory effect on the growth of SH-SY5Y cells (Fig. 4B).

## DISCUSSION

Sodium butyrate is the sodium salt of a short chain fatty acid, which is produced in the carbohydrate fermentation in bacteria [Blottiere et al., 2003]. Previous reports [Hara et al., 2000; Ruemmele et al., 2003; Bandyopadhyay et al., 2004; Louis et al., 2004; Cho et al., 2006; Mohana et al., 2007; Pajak et al., 2007; Lane and Chabner, 2009; Ma et al., 2009] have shown that sodium butyrate plays an important role in affecting cell maturation pathways such as cell cycle arrest, differentiation, and apoptosis. Many genes are transcriptionally regulated by sodium butyrate through histone hyperacetylation, which is resulted from the global suppression of histone deacetylation by sodium butyrate [Blottiere et al., 2003].

This study has demonstrated that sodium butyrate induced morphological changes (appearance of neuron-like long axis), and suppressed proliferations of SH-SY5Y neuroblastoma cells. It is well-known that sodium butyrate can significantly up- and down-regulate many genes that are related to cellular proliferation. We found a novel gene pp3501, as has been reported by Wan et al. [2004], was induced by sodium butyrate in SH-SY5Y cells (Fig. 1F,G); pp3501 mRNA levels increase in SH-SY5Y cells after 48 h exposure to 3 mmol/L sodium butyrate. The expression of pp3501 protein has also been confirmed by western blot with anti-pp3501 serum. Since pp3501 expression was related to sodium

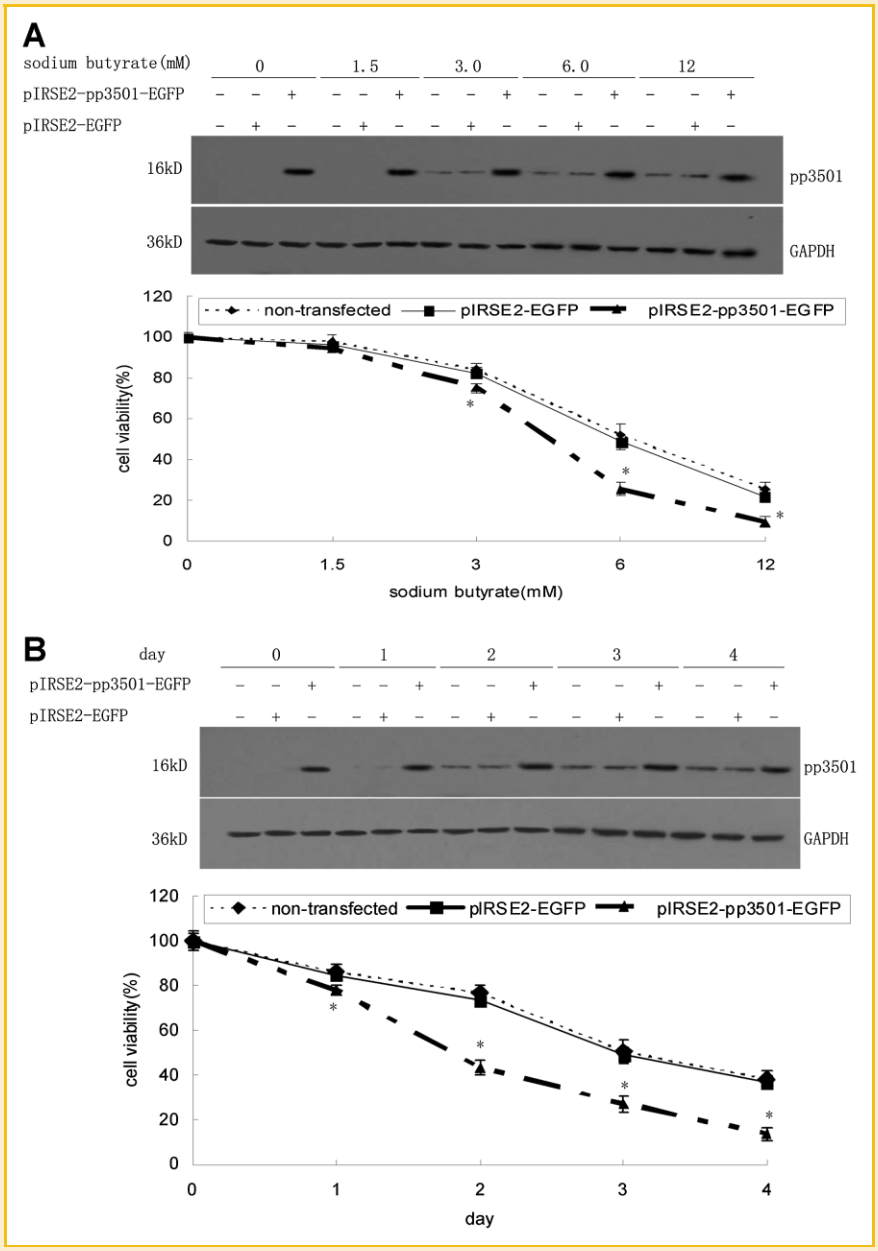


Fig. 3. pp3501 protein enhances the sensitivity of SH-SY5Y cells to sodium butyrate. SH-SY5Y cells, transfected with pIRSE2-pp3501-EGFP or pIRSE2-EGFP vectors, were treated with different concentrations of sodium butyrate for 24 h or treated with 3 mmol/L sodium butyrate for different times. Cell viabilities were determined by MTT assay. A: SH-SY5Y cells were treated with different doses of sodium butyrate for 24 h; (B) SH-SY5Y cells were treated with 3 mmol/L sodium butyrate for different times. The data were represented as mean ± SD ($n = 6$; $^{*}P < 0.05$ vs. pIRES2-EGFP-transfected group).

butyrate treatment, we speculated that pp3501 may have been involved in the antiproliferative activities of sodium butyrate. In addition, the expressions of pp3501 have been detected in other cell types including Raji cell, skeletal muscle stem cell, some hepatocellular carcinoma cells, lymphocyte, and normal tissue after sodium butyrate induction (data no shown). Wan et al. [2004] have identified pp3501 gene in their genome wide searches for cell growth-related genes by large-scale cDNA transfection method. Total 3,806 genes have been identified and recorded; one of them, located on chromosome 19, is named as pp3501.

It is surprising that pp3501 gene has an intact exon of expression frame without introns (according to Genbank), thus it may be one of those processed pseudogenes, which are commonly non-functional. However, our data have shown that pp3501 gene was induced by sodium butyrate and exerted inhibitory effect on the proliferation of SH-SY5Y cells. pp3501 gene encodes a 16 kDa protein with a pI of 11.6 (analyzed by ProtParam software). The analytical result from Hopfield neuron network has shown that pp3501 protein may contain three helical structures and six β-sheets.

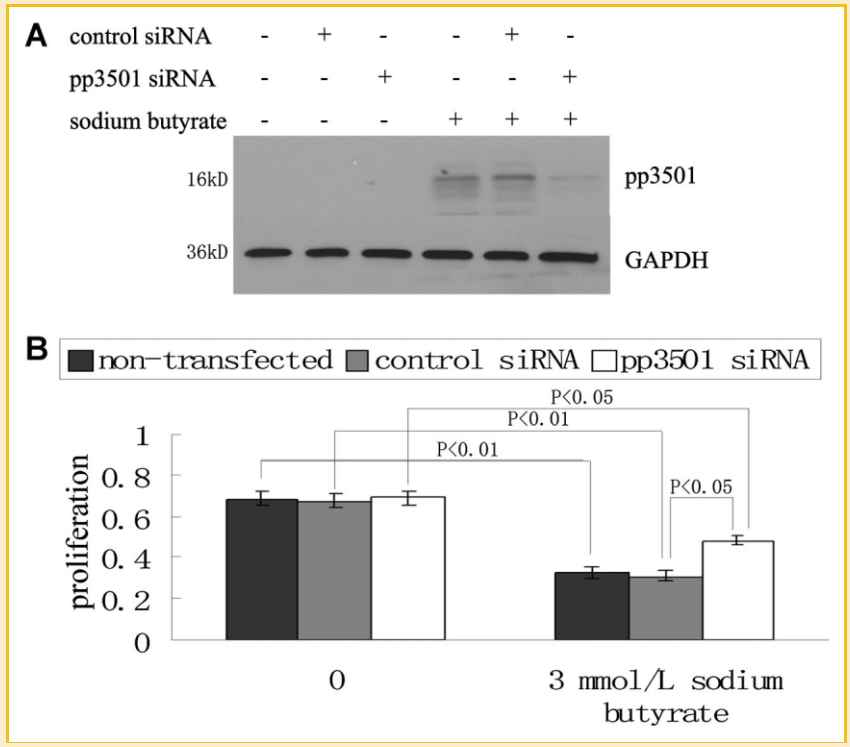


Fig. 4. pp3501 siRNA alleviates the inhibitory effect of sodium butyrate on SH-SY5Y cells. SH-SY5Y cells were transfected with pp3501 siRNAs for 24 h and were treated with 3 mmol/L sodium butyrate for another 48 h, and the cell viability was determined by MTT assay. A: pp3501 expression detected by western blot. B: Cell viability was determined by MTT assay. The data were represented as mean ± SD (n = 4; $^{*}P < 0.05$ vs. control siRNA-transfected group).

EGFP-pp3501 fusion protein was uniformly distributed in the nucleus, suggesting the nuclear localization of pp3501. However, green dot structures appeared in the nucleus, which may indicate that endogenous pp3501 mainly concentrated in the nucleolus (Fig. 1H). However, further experiments are needed to confirm whether the concentrated distribution of pp3501 in nucleolus. Since pp3501 localized in the cell nucleus, it may be involved in the regulation of sodium butyrate-induced gene expression.

pp3501 is a novel gene with unknown functions. We found that sodium butyrate upregulated pp3501 expression in SH-SY5Y cells. However, since the endogenous pp3501 expression was very low, we could detect the expression of pp3501 mRNA and protein in cells only after the sodium butyrate induction. Although sodium butyrate can induce cell apoptosis [Hara et al., 2000; Ruemmele et al., 2003; Bandyopadhyay et al., 2004; Mohana et al., 2007; Pajak et al., 2007], exogenous pp3501 alone did not appear to induce apoptosis. Therefore, cell cycle analysis has not shown the peak of subG1, one of the apoptotic markers. Sodium butyrate can induce the expression of many genes, and pp3501 is only one of them, which also explains why sodium butyrate-induced morphological changes cannot be fully resembled by pp3501 overexpression.

Proliferation of SH-SY5Y cells was inhibited by pp3501 expression. This coincides with the significantly suppressing effect of pp3501 on the proliferation of hepatoma cell SMMC-7721 described by Wan et al. [2004].

In addition, pp3501 expression sensitized SH-SY5Y cells to sodium butyrate-induced growth-inhibitory effect, indicating that the efficacy of sodium butyrate has been enhanced by pp3501 protein. Although siRNA knock-down of pp3501 did not completely antagonize the inhibitory effect of sodium butyrate on SH-SY5Y proliferation, it significantly alleviated the inhibition (Fig. 4B), indicating that although pp3501 is involved in the sodium butyrate-induced inhibition of SH-SY5Y cell growth, it is not indispensible for the action of sodium butyrate.

Moreover, pIRSE2-pp3501-EGFP has arrested the cell cycle of SH-SY5Y cells at G1 phase. Previous reports demonstrated that sodium butyrate inhibits cell proliferation by blocking cell cycle at G0/G1 phase [Pajak et al., 2007; Wang et al., 2008; Lane and Chabner, 2009]; therefore, we speculated that pp3501 may be the molecule mediating the inhibitory effects of sodium butyrate on cell cycle. Studies by Finzer et al. [2001] have demonstrated that sodium butyrate arrests human papillomavirus-positive carcinoma cells in G1 to S transition, which is paralleled by an up-regulation of p21CIP1 and p27KIP1 as well as the loss of cdk2 activity. However, it is still unknown whether pp3501 expression is involved in the induction or function of p21CIP1 and p27KIP1.

It has been demonstrated that sodium butyrate suppresses cell proliferation with the involvement of p53 [Bandyopadhyay et al., 2004], bcl-2 family [Hara et al., 2000; Ruemmele et al., 2003; Louis et al., 2004; Cho et al., 2006; Mohana et al., 2007; Pajak et al., 2007], and p21CIP1 and p27KIP1 [Finzer et al., 2001] proteins. Therefore, further investigation of the relationship of pp3501 with above-mentioned proteins forms the subject of our future studies.

In conclusion, this study has demonstrated that pp3501 protein is localized in the nucleus of SH-SY5Y cells and can be detected with specific rabbit antiserum. pp3501 inhibits the proliferation of SH-SY5Y cells by arresting the cell cycle at G1 phase. The present work not only sheds light on the mechanism by which sodium butyrate inhibiting cell proliferation but also provides a new direction for future studies of the function of this novel pp3501 protein and its potential role in tumor therapy.

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