RESEARCH ARTICLE

Fentanyl Increases Colorectal Carcinoma Cell Apoptosis by Inhibition of NF-*x*B in a Sirt1-dependent Manner

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Abstract

Background: Fentanyl is used as an analgesic to treat pain in a variety of patients with cancer and recently it has become considered to also act as an antitumor agent. The study present was designed to investigate the effects of fentanyl on colorectal cancer cell growth and plausible mechanisms. <u>Materials and Methods</u>: The human colorectal carcinoma cell line HCT116 was subcutaneously injected into nude mice. The viability of HCT116 was tested by MTT assay, and apoptosis by flow cytometry and caspase-3 activity. The expression of Sirt1 and NF-×B were evaluated by Western blotting and the levels of Sirt1 and NF-×B by fluorescence method. SiRNA was used to silence and Ad-Sirt1 to overexpress Sirt1. <u>Results</u>: Our data showed that fentanyl could inhibit tumor growth, with increased expression of Sirt1 and down-regulation of Ac-p65 in tumors. Compared with control cells without treatment, HCT116 cells that were incubated with fentanyl had a higher apoptotic rate. Moreover, fentanyl could increase expression and activity of Sirt1 and inhibitor expression and activity of NF-×B, which might be mechanisms of fentanyl action. <u>Conclusions</u>: Fentanyl increased colorectal carcinoma cell apoptosis by inhibition of NF-×B activation in a Sirt1-dependent manner.

Keywords: Colorectal carcinoma cells - fentanyl - NF-xB - Sirt1

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Introduction

Worldwide, colorectal cancer is one of the most prevalent and incident common malignant tumors, followed by lung and breast cancers, and it was the fourth leading cause of cancer death (Jemal et al., 2011). A prediction made by World Health Organization indicates that an increase in the number of newly diagnosed cases and deaths from colorectal cancer by 2030. Given this, there is an urgent need for research to prevent and treat this disease.

Fentanyl, a agonist for μ -opioid receptor, is a kind of strongly anesthetic analgesic drugs widely used in clinical (Nuckols et al., 2014). Especially, it is considered to be a good analgesic effective on cancer pain in terminal cancer patients. Recently, studies reported that fentanyl has a activity of inhibiting of cancer cell proliferation and cancer progression (Huffman et al., 2007; Qin et al., 2012), which suggested that a potential antitumor role of fentanyl. However, it remains unknown whether fentanyl has a sustain effect on colorectal cancer.

Sirt1 is a kind of NAD+-dependent Class III histone deacetylase and it play a multiple role in tumor suppression or oncogenesis, stress responses, cellular metabolism and aging through target its substrate (Yi and Luo, 2010). Presently, several paper reported that the relationship between Sirt1 and colorectal cancer involved in tumor malignant degree, transfer, patient survival rate and cancer cell proliferation (Kabra et al., 2009; Jung et al., 2013; Lv et al., 2014). Whether sirt1 acting as a cancer-promoting genes or a tumor suppressor gene is always a controversy.

As a deacetylase target of Sirt1, nuclear factor-kappa B (NF- κ B) regulate significant cell biological activity including cell cycle, apoptosis, adhesion and angiogenesis through interacting with its downstream genes (Domingo-Domenech et al., 2008). There has been reported that NF- κ B signaling pathway takes part in fentanyl inhibiting cancer cells proliferation (Yi and Luo, 2010). However, it remains a lack of adequate evidence to prove the relationship between fentanyl and NF- κ B. Our study was designed to investigate the relationship between fentanyl and colorectal carcinoma, and further preliminarily explore the molecular mechanism underlying it, which provides a potential clinical utility.

Materials and Methods

Cell lines, culture conditions and fentanyl treatment

HCT116 human colorectal carcinoma cells were purchased from China Centre for Type Culture Collection (Shanghai, China) and cultured in phenol red-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen,

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Carlsbad, CA, USA). Cells were subcultured every 3-5 days to maintain logarithmic growth until a sufficient number of cells (5×10^6 cells/ml) were obtained for transfer to nude mice. High purity fentanyl was purchased from Sigma (St. Louis, MO, USA), dissolved in DMSO and added into the culture medium at the indicated concentration. All experiments were carried out 24 h after fentanyl supplementation, unless otherwise indicated.

Tumor xenograft assay

Experimental procedures involving animals were reviewed and approved by the Institutional Animal Care and Use committee of the University of Zhejiang. Six week-old athymic nude mice were housed in filter-topped cages and received food and water freely. Tumors were generated by subcutaneous injection into the abdomen with 5×10^6 cells/ml of HCT116 cells suspended in 100 μ L PBS and mixed with 20% matrigel. After five days from the cell inoculated, fentanyl was injected into tumors in dose of 0.04mg/kg, 0.08mg/kg, 0.16mg/kg, 0.32mg/ kg (per two days) in three weeks. Thirty five days after inoculation, animals were euthanized and tumors were removed and weighted.

MTT Viability Assay

The effects of fentanyl on cell viability were determined in an MTT assay (Sigma-Aldrich, St. Louis, MO, USA), which is based upon 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

Bromide uptake.

Cells (2×10³/well) were seeded in 96-well plates 24 h prior to fentanyl treatment. After respectively 24h, 48h, 72 h of fentanyl treatment, MTT solution was added to each well and incubated for 2 h at 37°C. Cells were incubated with SDS Buffer (10%) with 0.01 M HCl overnight and the absorbance was measured at 570 nm using a spectrophotometer (PerkinElmer Inc, Boston, MA, USA). Independent experiments were performed three times in triplicate.

Caspase-3 activity assays

Caspase-3 activity was analyzed using the caspase-3 activity assay kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's protocol. Cells were lysed and total cellular protein extracts were quantified by BCA protein assay kit (Beyoutime, Jiangsu, China). And an equal amount of total protein extract was incubated at 37°C overnight with either acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA) for caspase-3 assay. The release of pNA was estimated by determining the absorbance at 405 nm on a microplate ELISA reader (Bio-Rad Laboratories). The relative activity of caspase-3 was calculated as follows: caspase-3 activity=(mean experimental absorbance/mean control absorbance)×100 (%).

Apoptosis analysis

Detection and quantitation of apoptosis were performed by flow cytometry. This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in early apoptotic cells. Briefly, cells were resuspended in a binding buffer. Annexin V-EGFP and PI were added and the solution was incubated at room temperature for 15 min in the dark, followed by assay on FACScan (Becton Dickinson). The percentage of apoptosis was computed using Cell-Quest software (Becton Dickinson).

HDAC enzymatic-activity assay

HDAC activity was analyzed using the HDAC Fluorimetric Activity Assay kit (Enzo life Sciences, CH) according to the manufacturers' instructions. HDAC activity values were corrected for non-specific (IgG)bound HDAC activity.

NF-κ*B* luciferase reporter assay

Cells or transfected Ad-Srit1 cells were plated in 35 mm dishes and 24 h later were transfected with NF- κ B-luciferase reporter plasmid (Aldevron, USA) using lipofectamine according to the manufacturers. At 6 h after transfection, cells were divided into equivalent aliquots and seeded in 24-well plates. After 18 h, cells were treated with the fentanyl or during 24 h. Ad-Srit1 cells directly were used for follow-up test. Then, cells were harvested and subjected to a luciferase assay by using the Luciferase Assay Kit (Promega, Madison, USA) according to the manufacturer's instructions. Relative light units were measured using Fujifilm LAS3000 Imaging system. The luciferase activities were normalized to protein contents and expressed as the fold increase relative to the activity of untreated controls.

Western blot analysis

Tumors or cells were lysed with ice-cold lysis buffer containing: 50 mmol/l Tris-HCl, pH 7.4; 1% NP-40; 150 mmol/l NaCl; 1 mmol/l EDTA; 1 mmol/l phenylmethylsulfonyl fluoride; and complete proteinase inhibitor mixture (one tablet per 10 ml; Roche Molecular Biochemicals, Indianapolis, IN, USA). Protein concentration in the cell lysate was quantified using the BCA protein assay kit (Beyoutime, Jiangsu, China). After determination of protein content, western blot analysis was performed. Proteins were separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was probed with Sirt1(1:200, SantaCruz, Santa Cruz, CA), Ac-p65(1:200, SantaCruz, Santa Cruz, CA), IkBa(1:200, SantaCruz, Santa Cruz, CA) or *p*-IkBa(1:200, SantaCruz, Santa Cruz, CA) followed by incubation with the secondary antibodies (1:1000, Sigma-Aldrich, St. Louis, MO, USA). The bands were visualized by enhanced chemiluminescence using ECL (Pierce Chemical) and captured on X-ray films.

Overexpression of Sirt1 by transfection

To generate stable cell lines, Sirt1 overexpression vector and the control vector (Open Biosystems, USA) were transfected into HCT116 cells using LipofectamineTM 2000. After 48 h, cells were trypsinized and reseeded into 10 cm culture plates. Cells were selected with puromycin at a final concentration of 2 μ g/ml for 3 weeks. Single colonies were selected, confirmed, and

expanded for the subsequent experiments.

Inhibition of Sirt1 by RNA Interference

Idouble-stranded oligonucleotide 5'-GATCCCG TTGGATGAT ATGACACTGTTCAAGAGACAGT GTCATATCATCCAACT TTTTTGGAAA (Sirt1 target sequence underlined) was cloned into the pSuperiorRetroPuro vector (Oligo Engine). The plasmid was packaged into retrovirus by transfection of the amphotropic packaging cell line LA (a kind gift from Dr. Peiqing Sun, the Scripps Institute). A virus expressing a scrambled shRNA (5'-GATCCCGCCGTCGTC GATAAGCAATATTTGATAT CCGATATTGCTTATCGACGACGGCTTTTTTA) was used as control. HCT116 cells were infected with the Sirt1 shRNA retrovirus and selected with 0.5 g/ml puromycin for 10 days. After that cells were incubated with fentanyl at 100 ng/ml for 24 h when necessary for subsequent test. Cells apoptosis and protein analysis were pooled for analysis.

Statistical analysis

Statistical analysis was conducted with statistical analysis software SPSS 13.0 software. Statistical analyses were performed by an analysis of variance (ANOVA) or Student's t-test. Data were expressed as mean \pm standard deviation. *p*<0.05 was considered to be statistically significant different.

Results

Fentanyl decreased the Growth of Colon Tumor Xenograft

To test the role of fentanyl in tumor formation, HCT116 colorectal cells were stably subcutaneously injected into the flank of the nude mice and then the tumors were injected with dosage of fentanyl at 0.04 mg/kg, 0.08



Figure 1. Inhibitor Effect of Fentanyl on Tumors Formation. Subcutaneously into nude mice to develop implantation tumor, and then injected dosages of fentanyl into tumor. The tumors were removed and analyzed (A) the weight of tumor and (B) the expression of Sirt1 and Acp65 in tumor. Results represent the mean \pm SD (*p<0.05, compared with control)

Apoptosis by Inhibition of $NF \times B$ in a Sirt1-dependent Manner mg/kg, 0.16 mg/kg and 0.32mg/kg. Three weeks later, the weight of tumor weight were decreased by fentanyl in a manner of concentration-dependent (Figure 1A). However, there was no significantly distinction between 0.04 mg/kg group and control group.

The expression of Sirt1 and acetylated p-65 (Ac-p65), which is a subunit of NF- \varkappa B, were also determined by Western blot in formated tumor respectively. As shown in Figure 1B, the result showed that fentanyl up-regulated in Sirt1 and down-regulated in Ac-p65 expression and the changes were also in a concentration dependent manner.

Fentanyl promoted Colorectal Carcinoma Cell Apoptosis

In order to define how fentanyl inhibit tumor formation, HCT116 were used to detect colorectal carcinoma cells apoptosis in vitro. When HCT116 were incubated with fentanyl at 0.5 g/ml, 5 ng/ml, 50 ng/ml, 100 ng/ml for 24 h, 48 h and 72 h, cells viability was lowest at 100 ng/ml of fentanyl for 72 h and declined in a dose and time-dependent manner (Figure 2A). Furthermore, flow cytometry result showed that cells apoptosis obviously increased after treated with fentanyl for 72 h (Figure 2B). In addition, fentanyl increased caspase-3 activity level of cells in a dose-dependent manner (Figure 2C).



Figure 2. Effect of Fentanyl on Colorectal Carcinoma Cell Viability and Apoptosis in HCT116. HCT116 cells were incubated with different concentration of fentanyl (0.5 g/ml, 5 ng/ml, 50 ng/ml and 100 ng/ml). (A) MTT analysis on cells inhibition rate in 24 h, 48h and 72h. (B) Annexin V-PI staining analysis on the apoptosis-inducing activity of fentanyl in HCT116 cells (treated with fentanyl for 72 h)with flow cytometry. (C) Analysis of caspase-3 activation in HCT116 cells (treated with fentanyl for 72 h) were performed. IC50=55.57 ng/ml for 72 h. Ac-p65, acetylated p65. Results represent the mean \pm SD (*p<0.05, compared with control)

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Fentanyl increased activation of Sirt1 and decrease transcriptional activity of p65 in HCT116

As showed in Figure 3A, the expression of Sirt1 was increased and the Ac-p65 expression was decreased in HCT116 cells by fentanyl treatment in a dose and time-dependent manner. This is in accordance with result of tumor xenograft. While, $I-\kappa B$, an inhibitor for NF- κB by binding to p65, and its phosphorylated level had no change(Figure 3A) at any dose of fentanyl.

Furthermore, we tested the decetylation activity of Sirt1 and the transcriptional activity of NF- κ B. Results showed that the increased decetylation activity of Sirt1 and the decreased transcriptional activity of p65 were both in a dose-dependent manner(Figure 3B, C).

Sirt1 overexpression suppresses NF-кВ signaling pathway

To further definite the effect of Sirt1 on NF-KB signaling pathway, adenoviral vector Sirt1 was transfected into HCT116 cells. As shown in Figure 4A, cells transfected with Ad-Sirt1 showed overexpression



Figure 3. Effect of Fentanyl on Sirt1 and NF- α B Activation in HCT116. Cells pre-incubated with fentanyl (0.5 ng/ml, 5 ng/ml, 50 ng/ml, 100 ng/ml) for 24 h and then analyzed the expression of Sirt1, Ac-p65, *p*-I α B α (A), Sirt1 deacetylation activity with HDAC fluorescence activity (B) and NF- α B activity with luciferase assay (C). Results represent the mean±SD (**p*<0.05, compared with control)



Figure 4. Effect of Overexpression of Sirt1 on NF- α B Transcriptional Activity. Cells were transfected with Ad-Sirt1 and then lysed to detect (A) p65 acetylation level and (B) NF- α B activity with luciferase assay. Ad-Sirt1, adenovirus vector Sirt1. Results represent the mean±SD (*p<0.05, compared with control)

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Figure 5. Effect of Fentanyl on NF- \approx B Transcriptional Activity and HCT116 Apoptosis after Silence of Sirt1. Cells were Transfected with Sirt1-siRNA for Experiment. The cell lysed to detect (A) p65 acetylation level and (B) caspase-3 activity. (C) Annexin V-PI staining analysis on cells apoptosis. NC, negative control; si-Sirt1, si-RNA on Sirt1. Results represent the mean±SD (*p<0.05, compared with control or NC, #p<0.05, compared with fentanyl group)

of Sirt1 and declined expression of Ac-p65 compared with cells without Ad-Sirt1 treatment. At the same time, the transcriptional activity of NF- α B was attenuated companied by Sirt1 overexpression (Figure 4B). The results supported that fentanyl activate Sirt1 to negative regulating NF- κ B signaling pathway.

Sirt1 silence reversed the effect of fentanyl on HCT116

To better demonstrated the role of Sirt1 expression on fentanyl induced cells apoptosis, HCT116 cells were infected with the Sirt1 siRNA retrovirus. The results demonstrated that downregulated Sirt1 reversed fentanyl induced increase in Sirt1 expression and decrease in Acp65 expression(Figure5A). On the other hand, fentanyl increased the level of apoptotic protein caspase-3 activity and si-Sirt1 attenuated the effect of fentanyl on caspase-3 activity in HCT116 (Figure 5B). In addition, cell apoptosis treated with fentanyl was also largely inhibited by si-Sirt1(Figure 5C).

Discussion

In the present study, we demonstrated that fentanyl inhibited tumor growth in nude mice subcutaneously injected with HCT116, as well as promoted colorectal carcinoma cell apoptosis. Further, fentanyl-promoted cells apoptosis via activation of Sirt1 and inhibition of NF-xB signaling pathway.

Several anesthesia and analgesia, which including fentanyl have been taken into account in the recurrence and metastatic rate of malignancies recently (Bovill, 2010; Lennon et al., 2012). Fentanyl with analgesic activity are widely used in clinic as anesthetics especially in cancer. Recent studies reported that fentanyl also played a role in inhibiting tumor. Qin et al. found that fentanyl inhibited

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proliferation of gastric cancer cells by down-regulated NF- α B and up-regulated Pten (Yi and Luo, 2010). Frances et al. suggested the mu opioid receptor (MOR) plays a crucial role in the fundamental cellular epithelial mesenchymal transition changes that occur during lung cancer progression (Lennon et al., 2014). Our research support the antitumor role of fentanyl, in which inhibiting tumor growth and proliferation but promoting colorectal cancer cells apoptosis in vitro. Furthermore, it was the first to get detect that fentanyl affected Sirt1 gene expression in colorectal carcinoma cell.

Several studies have focused on the relationship between Sirt1 and cancers. Certain observations have indicated that the depletion of Sirt1 by small interfering RNA (siRNA) induces tumor cell death with no toxicity on normal cells (Ford et al., 2005). A previous study has provided strong evidence that Sirt1 is significantly overexpressed to function as a tumor promoter in mouse and human prostate cancer (Huffman et al., 2007). But, in our research, fentanyl increased Sirt1 expression accompanied by inhibition of tumor formation and promotion of cancer cell apoptosis. And Sirt1 gene recombinant adenovirus vector transfection result indicated that Sirt1 overexpression also lead to cell apoptosis in HCT116 cell. This results suggested a anti-tumour role of Sirt1 which activated by fentanyl. The result is in accordance with previous studies, in which indicated is an inhibitor of Sirt1 in colon cancer (Yeung et al., 2004; Kabra et al., 2009). These seemingly contradictory roles show that Sirt1 has a complicated function in tumorigenesis, in which as a result of experimental conditions, object of study or else.

As a target gene of Sirt1, NF-xB has been found to be constitutively active in a number of solid tumors and hematological malignancies, and it is also changed in many different tumor cell lines (Xu et al., 2013; Gang et al., 2013). And its activity was performed in the expression of genes that participate in tumor promotion, angiogenesis and metastasis and resistance to various chemotherapeutic agents (Aggarwal, 2004; Domingo-Domenech et al., 2006). In our result, in addition of Sirt1 activation, fentanyl also inhibited the expression and activity of NF-xB in tumor and HCT116 cell. The regulating in transcriptional activity of NF-xB is focused on posttranslational modifications, such as phosphorylation and acetylation (Chen and Greene, 2004). It has been reported that Sirt1 could deacetylate p65 subunit of NF-xB at lysine 310 to inhibit its transcription physically (Lee et al., 2009; Lv et al., 2014). This study suggested that increased expression and activation of Sirt1 accompanied by decreased that of Ac-p65 in vivo and cells under the influence of fentanyl. It implied a regulated role of Sirt1 on NF-xB signaling pathway in fentanyl treating HCT116 cell. And it was confirmed by overexpression of Sirt1 and no effect on expression and activity of IxB by febtanyl which ruled out biological function of IzB in this process. Complementally, IxB, inhibitor of NF-xB, bound to NF-xB complexes resulting in its sequestering in the cytoplasm in non-stimulated cells. And when expose to a variety of extracellular stimuli, IxB phosphorylated by IxB kinase (IKK) resulting in its degradation and regaining activity of NF-xB (Ghosh et al., 1998; Karin et al., 2004).

To further ascertain that fentanyl-promoted colorectal carcinoma cells apoptosis via activation of Sirt1 related signaling transfer, Sirt1 was silenced. It leaded to the role of fentanyl in HCT116 cell reversed. In summary, our result suggested that in addition to other biological activities, fentanyl has also play an important role in inhibiting tumor growth via Sirt1. In our preliminary discussion, fentanyl induced cell apoptosis by restraining activity of NF-xB mediated by Sirt1 activation.

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