

## Effects of Fentanyl on pancreatic cancer cell proliferation and cancer stem cell differentiation

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**Abstract:** Pancreatic cancer is one of the most aggressive cancer due to the late diagnosis and failure to respond to the treatment despite advances in tumor biology and the development of new cancer therapeutic strategies. It has been reported that these characteristics of pancreatic cancer originate from cancer stem cells within the tumor mass. It has also been reported that Fentanyl is a fast-acting analgesic that binds to the mu-opioid receptors and some other mu-opioid receptors are involved in this cancer process. In this study, we determined the effect of Fentanyl on PANC-1 cells, by assessing the gene expression of cancer stem cell marker genes (*Nanog*, *Oct4*, and *Sox2*) and apoptosis-related genes (*BAD*, *Bax*, *Bcl-2*, and *p53*) by Quantitative RealTime PCR. The number of cancer stem cells was determined by Flow Cytometry. The results of our study showed that Fentanyl administration decreased the number of cancer and cancer stem cells in the PANC-1 cell population, decreased the gene expression of stem cell marker and increased the expression of apoptosis-related genes. These results indicate that Fentanyl, which is used routinely in the pain palliation of pancreatic cancer, can be considered as an option in the treatment of pancreatic cancer.

**Key words:** Apoptosis; Cancer stem cell; Fentanyl; PANC-1 cell.

### Introduction

Pancreatic cancer is one of the most leading cause of death among all cancers and the 5-year survival rate of this cancer is between 1-5% (1). The diagnosis of pancreatic cancer is usually delayed, metastases occur early stage and this leads to a high patient mortality rate (2). In some publications, pancreatic cancer named as 'silent killer' because it is very aggressive and has a little expectancy of treatment (3). The most effective treatment for today is cytotoxic chemotherapeutic applications (1). This may be convenient only for the patients diagnosed at an early stage of the disease and the patients positively respond to the treatment (1). Although the cause of the aggressiveness of pancreatic cancer is not known exactly, it is thought that one of the reason is the cancer cells that have stem cell characteristics within the tumor mass (4). These cells maintain the continuity of cancer cells and also they are important in the development of drug resistance to the chemotherapeutics (5). Drug resistance in pancreatic cancer patients is one of the main causes of failure in the treatment of pancreatic cancer. Studies indicate that drug resistance in pancreatic cancer is due to tumor microenvironment interactions, changes in signaling pathways and cancer stem cells, which are highly resistant to chemo-radiotherapy (6). According to the Cancer Stem Cell model, the main mass of the tumor does not only consist of differentiated cells, but tumor mass also contains a small number of stem cells, which are called Cancer Stem Cells (CSC)

(7,8).

In the treatment of pancreatic cancer, the tumor mass can be regressed, but the cancer stem cells in this population maintain the strain and they are leading to relapse, and multi-drug resistance to the chemotherapeutic drug (9). It has been shown that CSCs in pancreatic tumor mass increases the aggressiveness of the tumor (10) and CD24 and CD44 stem cell markers increase with the increased aggressiveness of the tumor (6,11). In a comprehensive study that differentially expression of more than 1500 genes was studied, a positive correlation was found between tumor aggressiveness and cancer stem cells (12). Suzuki et al. reported that the cancer cell population continuing cancer stem cells were resistant to 5-FU and Gemcitabine more than the population without cancer stem cells (13).

Studies reveal the importance of cancer stem cells within the tumor mass in the occurrence, persistence, drug resistance and aggression of pancreatic cancer. The elimination of these stem cells can contribute to the treatment of cancer. There are some reports that antagonist of mu-opioid receptors has a positive effect on cell differentiation or elimination of stem cells (14,15). Fentanyl is a fast-acting analgesic that binds to the mu-opioid receptors and frequently used in patients with pancreatic cancer (16). In this study, we aimed to investigate the effects of fentanyl on stem cell differentiation and apoptosis in PANC-1 cells, due to its effects on cell differentiation and frequent use in pancreatic cancer.



## Materials and Methods

### Cells and reagents

The pancreatic adenocarcinoma cells, PANC-1 cell line and human embryonic kidney cell line, HEK-293 were purchased from American Type Culture Collection (ATCC, Rockville, Maryland 20852, USA), and both cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Sigma-Alrich, St. Louis, MO) and 1% antibiotic-penicillin solution (Sigma-Alrich, St. Louis, MO) at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>. Cells were harvested with 0.25% trypsin (Sigma- Aldrich, St. Louis, USA) after the cell culture reached 80% confluency. High purity Fentanyl was purchased from Sigma (St. Louis, MO, USA) and a 10% solution prepared with DMSO, various final concentrations of Fentanyl carried out with media and cells were treated with these various concentrations of Fentanyl in different time durations.

### The IC50 dosage of Fentanyl determined by MTT assay

To determine the IC50 dose of Fentanyl, approximately 1X10<sup>4</sup> cells were seeded into the 96 well-plate and cultured for 24 hours. Then, the predetermined doses of Fentanyl (10, 15, 20 and 25 μM) were applied to the wells and the cells were cultured for 24, 48 and 72 hours. In each dose group, a well was cultured under the same conditions as the control group without Fentanyl administration. After 24, 48 and 72 hours of the application, the cells in each well subjected to a Cell Proliferation Kit (MTT, Sigma-Alrich, St. Louis, MO), according to the manufacturer's instructions and absorbance values were quantified at 490 nm in a microplate reader.

### Fentanyl application and isolation of stem cells

After the determination of the active dose of Fentanyl by MTT assay, PANC-1 and HEK-293 cells were cultured with 25 μM of Fentanyl for 48 hours. Then approximately 10,000-12,000 cells were removed by trypsin-EDTA and centrifuged at 1500 rpm for 5 min to subject to stem cell isolation. The cellular pellets of

PANC-1 and HEK-293 cells were treated with CD24 and CD44 antibody, SSEA-1 and SSEA-4 (BD, Pharmingen®) respectively, in dark in 1X HBSS solution prepared with 2% FBS medium for half an hour. The control groups were diluted with 1% HBSS solution with 2% FBS. The CD44+/CD24+ and SSEA-1/SSEA-4 cells were sorted by FACS device (FACS Aria III - BD Pharmingen, USA) as stem cells in the cultured cell population and used in further studies.

### In vitro tumorsphere formation assay

In this study, stem cells were sorted from PANC-1 and HEK-293 cells by using specific antibodies against the cell surface markers. The isolated cells from the cell lines were cultured in serum-free medium. The spherical formations occurred after 45 days, thus cells were confirmed to be the stem cells by spheres formation technique.

### RNA isolation and the quantitative real-time polymerase chain reaction

The Fentanyl-treated and untreated cell groups, including sorted stem cells, were treated with TRIzol (Sigma Aldrich®), chloroform and isoamyl alcohol. RNA was precipitated with 75% ethyl alcohol and dissolved in RNase-free water. Concentrations of RNAs were measured at 260 and 280 nm by NanoDrop 2000 (Thermo Fisher Scientific). cDNAs were synthesized by using 1 μg of total RNA (RevertAid First Strand cDNA Synthesis, Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed in triplicate with the 7500 Fast Real-time PCR instrument (Applied Biosystems). The nucleotide sequences of the primer pairs used for Quantitative gene expression were given in Table 1. For determine the state of apoptosis before and after Fentanyl administration *Bad*, *Bax*, *Bcl-2*, and *p53* gene expression levels were studied. The expression levels of *Nanog*, *Oct4* and *Sox2* genes were determined to specify the effect of Fentanyl on cell differentiation. *GAPDH* gene used for normalization. The comparative ΔCT method was used to calculate the relative quantification

**Table 1.** The nucleotide sequences of the primer pairs used for Quantitative gene expression.

Primer	Oligonucleotide sequence (5'-3')	Location	Amplicon size (bp)
<i>BAD F</i>	5'-CCC AGA GTT TGA GCC GAG TG-3'	12-31	249
<i>BAD R</i>	5'-CCC ATC CCT TCG TCG TCC T-3'	260-242	
<i>BAX F</i>	5'- CCC GAG AGG TCT TTT TCC GAG-3'	116-136	155
<i>BAX R</i>	5'- CCA GCC CAT GAT GGT TCT GAT-3'	270-250	
<i>BCL2 F</i>	5'-GGT GGG GTC ATG TGT GTG G-3'	460-478	89
<i>BCL2 R</i>	5'-CGG TTC AGG TAC TCA GTC ATC C-3'	548-527	
<i>P53 F</i>	5'-CAG CAC ATG ACG GAG GTT GT-3'	382-401	125
<i>P53 R</i>	5'-TCA TCC AAA TAC TCC ACA CGC-3'	506-486	
<i>OKT4 F</i>	5'-TGC CTC AGT ATG CTG GCT CT-3'	44-63	193
<i>OKT4 R</i>	5'-GAG ACC TTT GCC TCC TTG TCC-3'	236-216	
<i>NANOG F</i>	5'-AAG GTC CCG GTC AAG AAA CAG-3'	268-288	237
<i>NANOG R</i>	5'-CTT CTG CGT CAC ACC ATT GC-3'	504-485	
<i>SOX2 F</i>	5'-GCC GAG TGG AAA CTT TTG TCG-3'	229-249	155
<i>SOX2 R</i>	5'-GGC AGC GTG TAC TTA TCC TCC T-3'	383-362	
<i>GAPDH F</i>	5'- GGAGCGAGATCCCTCCAAAAT-3'	108-128	197
<i>GAPDH R</i>	5'-GGCTGTTGTCATACTTCTCATGG -3'	304-282	

of gene expression (17).

### Statistical analysis

Statistical analyses were performed by using SPSS 21.0 software. Comparisons between treated groups and untreated controls were carried out by using the paired t-test, one-way ANOVA test, and Tukey-Kramer post-test. Data were expressed as means ± SEM. Fold changes in gene expression using the comparative ΔCT method and statistical analysis were determined using the freely available Relative Expression Software Tool (REST 2009, Qiagen). The tests considered a basic significance level of p < 0.05.

### Results

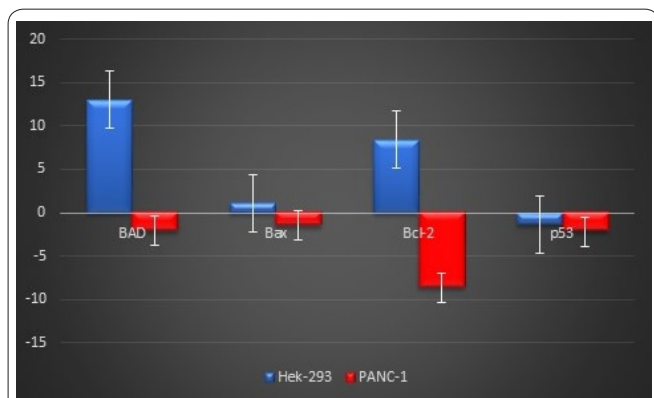
#### Effect of Fentanyl on PANC-1 cells and determination of IC50 dose

Effect of Fentanyl on PANC-1 cells determined by the administration of various concentrations of Fentanyl (10, 15, 20 and 25 μM) to cultured PANC-1 cells for 24, 48 and 72 hours. Cell viability assessed by the MTT assay. Results indicate that Fentanyl inhibited the cell proliferation of PANC-1 cells. The IC50 dose of Fentanyl was determined as 25 μM for 48 h.

#### Quantitative gene expression results of PANC-1 and HEK-293 cells

To assess the effect of Fentanyl on the apoptosis pathway, the gene expression levels of apoptosis-related genes *BAD*, *Bax*, *Bcl-2* and *p53* were determined both in PANC-1 and HEK-293 cells. Relative expression levels were determined after the normalization with *GAPDH*. According to the results, Fentanyl administration increased the expression of *BAD*, *Bax* and *Bcl-2* genes in HEK-293 cells, however, decreased in PANC-1 cells. Gene expression of *p53* decreased in both cell types but decreased much more in PANC-1 cells. There were statistically significant differences between the gene expressions of *BAD*, *Bax* and *Bcl-2* (p<0.001). Results summarized in Figure 1.

In this study, we evaluated the gene expression levels of stem cell markers such as *Nanog*, *Oct4*, and *Sox2* in both cell lines. According to the results, gene expression levels of *Nanog* and *Sox2* increased in HEK-293 cells however decreased in PANC-1 cells. The expression of

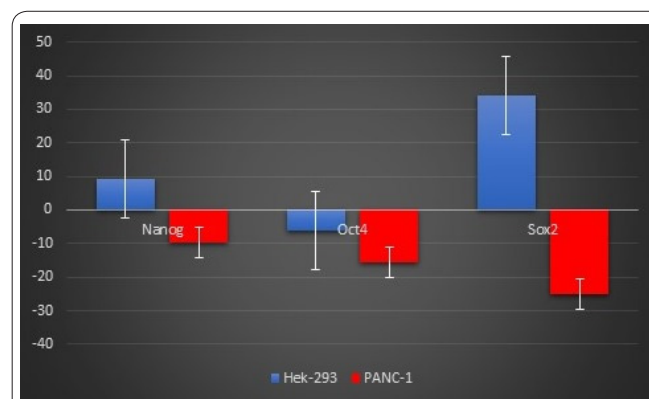


**Figure 1.** Representation of apoptosis-related gene expressions. Gene expression of *BAD*, *Bax* and *Bcl-2* were increased in HEK-293 cells and decreased in PANC-1 cells. The expression of *p53* was decreased in both cell type.

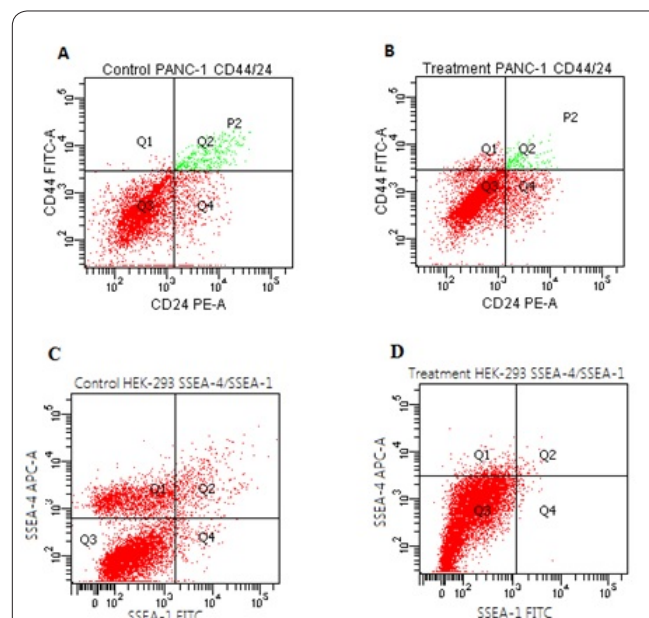
*Oct4* gene decreased both cell types. There was a statistical difference between Fentanyl treated and untreated PANC-1 cells for three genes (p<0,05). Results summarized in Figure 2.

#### Quantification of stem cells by flow cytometry

The effect of Fentanyl on stem cells analyzed by the stem cell markers CD44 and CD24 for PANC-1 cells and SSEA-1 and SSEA-4 for HEK-293 cells. According to results, Fentanyl decreased the percentage of CSCs in the PANC-1 cell population. The stem cell percentage of control of PANC-1, without Fentanyl administration, was 3.2, however, it was 1.5% in Fentanyl treated group. In HEK-293 cells, the percentage of CSCs increased after the Fentanyl treatment. Results are shown in Figure 3.



**Figure 2.** Representation of gene expression of stem cell markers. The gene expression levels of *Nanog* and *Sox2* were increased in HEK-293 cells and decreased in PANC-1 cells. Expression of *Oct4* was decreased in both cell types.



**Figure 3.** Cancer stem cell quantitation of PANC-1 and HEK-293 cell. Part (A) represents the amount of cancer stem cells in untreated PANC-1 cells, it was found 3.2%. Part (B) represents the amount of cancer stem cells after the Fentanyl treatment, it was found 1.5%. Part (C), Quadrant 3 represents the amount of SSEA-1+ and SSEA-4+ HEK-293 cells, it was found 56.8%, Part (D), represents the amount of SSEA-1+ and SSEA-4+ HEK-293 cells, it was found 96,4%.



## Discussion

Pancreatic cancer is a highly aggressive type of cancer with a 5-year survival rate of less than 5% (4). The reason for this aggressiveness and resistant to chemoradiotherapy is not known exactly, but it is thought to be caused by cells that exhibit stem cell characteristics within the tumor mass. In several studies conducted in pancreatic cancer, it has been shown that cancer stem cells play an important role in both endothelial-mesenchymal transmissions of pancreatic cancer and resistance to chemotherapeutics (18). A positive correlation was found between the tumor's aggressiveness and cancer stem cells by Bao et al. in their study that they have studied differentially expression of more than 1500 genes (12). Shah et al. reported the increased expression of CD24 and CD44 in gemcitabine-resistant pancreatic cells and also they found a relationship between the cancer stem cells, the aggressiveness of pancreatic cancer, and gemcitabine resistance (6,11). In the study we performed in PANC-1 cells, we determined the percentage of stem cells as 3.2% in the total cancer cell population. The percentage obtained from HEK-293 cells was 56.8%. This rate is quite high for PANC-1 when it compared to the knowledge from the literature (4). These studies reveal the importance of cancer stem cells within the tumor mass in the occurrence, persistence, and aggression of pancreatic cancer.

In accordance with the findings in the literature, our results showed that the number of stem cells in the pancreatic cancer cell population was higher. The elimination of these cells or induction to differentiation is important in preventing pancreatic cancer. It has been shown that mu-opioid receptors are involved in stem cell differentiation (19,20). Fentanyl is an opioid analgesic which is used in cancer patients wherefore it is 80 times more effective than morphine (21). At the beginning of this study, we aimed to determine the effect of Fentanyl, as an opioid drug, on cancer stem cells. Flow cytometry results showed that the number of CSCs were decreased after Fentanyl administration in PANC-1 cells. However, the number of stem cells increased in non-tumor HEK-293 cells. After these findings, we determined the gene expression levels of stem cell markers in both cell type before and after Fentanyl administration. Our results showed that the expression of *Nanog*, *Oct4* and *Sox2* genes, which are stem cell markers, decreased significantly in PANC-1 cells after Fentanyl administration. In HEK-293 cells, the expression of *Nanog* and *Sox2* genes were increased. The expression of *Oct4* decreased in both cell types, but the decrease in PANC-1 cells was much higher. These results suggest that Fentanyl affects the cells that have stem cell characteristics in the pancreatic cancer cell population. Both flow cytometry results and the results of the expression of stem cell markers indicate that Fentanyl leads to differentiation or apoptosis of pancreatic cancer stem cells.

After determining the effect of Fentanyl on CSCs, we thought to study the situation of apoptosis after Fentanyl treatment. To determine the effect of Fentanyl on the apoptosis pathway, we studied the expression levels of *BAD*, *Bax*, *Bcl-2* and *p53* genes which are apoptosis markers, in Fentanyl-treated and untreated cells. We also determined the expression levels of the same genes

in HEK-293 cells to evaluate the effect of Fentanyl on non-tumor cells. The results showed that the expression of *BAD*, *Bax*, *Bcl-2* and *p53* genes decreased in PANC-1 cells after Fentanyl administration. We determined that the expression of these genes, except *p53*, increased in HEK-293 cells after Fentanyl administration. In a study conducted by Kocak et al. they found a positive correlation between Fentanyl treatment and differentiation of cancer stem cells and induction of apoptosis in MCF-7 breast cancer cells (22). The positive induction of Fentanyl on apoptosis was shown by Sabir et al. in their study conducted on newborn pigs (23). Wang et al. showed that the Fentanyl increases the *miR-302b* expression and inhibits proliferation and invasion by enhancing the expression of this microRNA (24).

In this study, we found that administration of Fentanyl reduces the number of cancer stem cells in the PANC-1 cell population and reduces the expression of the stem cell markers, *Nanog*, *Oct4* and *Sox2* genes. In the HEK-293 cells, we included in the study to see how the Fentanyl affects normal cells, as opposed to PANC-1 cells Fentanyl increased the number of the stem cells and also increased the apoptosis-related genes, except *p53*. According to the results obtained from the cell culture, Fentanyl can be used not only as an analgesic in pancreatic cancer but also it can be considered as a treatment option.

## Conflict of interests

The authors declare no conflict of interests.

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## Authors' Contributions

Collected, analysed and interpreted data, wrote the manuscript: Feyzi Celik; Tugce Duran. All authors read and approved the manuscript.

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