

Original Article

Activation of NPY1R increases the proliferation and invasion of human uterine leiomyosarcoma cells

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Abstract: Background: Uterine leiomyosarcoma is malignant tumor that arises from smooth muscle lining the walls of uterus. Neuropeptide Y Receptor Y1 (NPY1R) is a novel peripheral blood marker predictive of metastasis and prognosis in breast cancer. However, it is unclear about how hormones affect the expression, and how NPY1R affects cell proliferation and invasion of uterine leiomyosarcoma cells. Therefore, the current study aimed to examine effects of estradiol and progesterone on NPY1R expression in uterine leiomyosarcoma, and effects of NPY1R on the proliferation and invasion of human uterine leiomyosarcoma cells. Methods: Expression of NPY1R, estrogen receptor and progesterone receptor in uterine leiomyosarcoma tissue and normal surrounding tissue from patients were detected by immunohistochemistry staining. Human uterine leiomyosarcoma SK-UT-1 cells were treated with estradiol (10 nM, 100 nM, 1 μ M and 2 μ M), and progesterone (10 nM, 100 nM, 1 μ M and 2 μ M) for 24 h respectively, and expression of NPY1R was detected by qPCR. SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μ M and 10 μ M) for 24 and 48 h, respectively. SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μ M) for 24 and 48 h. The cell viability of SK-UT-1 cells was measured by CCK-8 cell viability assay kit. The proliferation of SK-UT-1 cells was measured by BrdU method. Transwell invasion assay was utilized to detect tumor invasion ability. Expression of matrix metalloproteinase (MMP) 2 and 9 was evaluated by qPCR. Results: NPY1R was expressed in human uterine leiomyosarcoma tissue. Compared to non-treated SK-UT-1 cells, estradiol at higher dosages (1 and 2 μ M) markedly increased NPY1R expression. Progesterone significantly increased NPY1R expression at all dosages used in current experiments. In addition, NPY1R agonist significantly increased viability of SK-UT-1 cells at higher dosages (100 nM, 1 μ M and 10 μ M) at 48 h. NPY1R antagonist markedly decreased viability of SK-UT-1 cells at higher dosages (10 nM at 24 h; 1 and 10 nM at 48 h). NPY1R agonist significantly increased the percentages of BrdU+ cells at higher dosages (100 nM, 1 μ M and 10 μ M) at 24 h. Meanwhile, NPY1R agonist markedly increased the invasion ability of SK-UT-1 cells dose-dependently, and NPY1R antagonist significantly decreased the invasion ability of SK-UT-1 cells in presence of NPY1R agonist. Compared to blank group, NPY1R agonist significantly increased the expression of MMP2 in SK-UT-1 cells dose-dependently at 48 h. Conclusions: Estradiol and progesterone can increase NPY1R expression in human uterine leiomyosarcoma cells. NPY1R agonist significantly increased proliferation and invasion of human uterine leiomyosarcoma cells, and NPY1R antagonist markedly decreased the proliferation and invasion, especially in presence of NPY1R agonist.

Keywords: NPY1R, uterine leiomyosarcoma, SK-UT-1 cells, proliferation, invasion

Introduction

Uterine leiomyosarcoma is malignant tumor that arises from smooth muscle lining the walls of uterus. It may develop in approximately one to five out of every 1,000 women with fibroids. Uterine leiomyosarcoma is a resistant cancer that generally not very responsive to chemotherapy or radiation. The 5-year survival rate is only 50% in patients whose tumor is confined to uterus [1]. Therefore, it is of great importance to investigate pharmacological approach-

es that may alter tumor characteristics such as proliferation and invasion, in order to improve therapeutic effectiveness against human uterine leiomyosarcoma cells.

Neuropeptide Y receptors are a class of G-protein coupled receptors that are activated by neuropeptide Y, peptide YY and pancreatic polypeptide. Neuropeptide Y receptors control various behavioral processes such as appetite, circadian rhythm, anxiety, and depression [2-5]. Activated neuropeptide receptors release Gi

subunit from the heterotrimeric G protein complex, which inhibits the production of second messenger cAMP from ATP. There are five known mammalian neuropeptide Y receptors: Y1, Y2, Y3, Y4, and Y5. Like other neuropeptide Y receptors, Neuropeptide Y Receptor Y1 (NPY-1R) regulates behavioral processes including autism and appetite [6, 7]. Naturally occurring genetic variation at NPY1R locus was reported to be implicated in heritable autonomic control of the circulation, and systemic hypertension [8]. In addition, NPY1R was recently revealed to be a novel peripheral blood marker predictive of metastasis and prognosis in breast cancer patients. High levels of NPY1R expression were positively correlated with clinical stage and lymph node metastasis status of breast cancer, as well as with the status of estrogen and progesterone receptors in breast cancer tissue. Breast cancer patients with circulating cancer cells that expressed NPY1R exhibited shorter tumor-specific survival when compared with those with no NPY1R expression [9]. However, it is unclear about whether NPY1R is expressed in uterine leiomyosarcoma, and how hormones such as estradiol and progesterone affect the expression. Meanwhile, effects of NPY1R on cell proliferation and invasion of uterine leiomyosarcoma cells require further investigations.

Therefore, the current study aimed to examine effects of estradiol and progesterone on NPY1R expression in uterine leiomyosarcoma, and effects of NPY1R on the proliferation and invasion of human uterine leiomyosarcoma cells.

Material and methods

Cells and reagents

SK-UT-1 human uterine leiomyosarcoma cell line was obtained from the Cell Bank of the Chinese Academy of Science (Shanghai, China). SK-UT-1 cells were cultured in RPMI-1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS; Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1% penicillin and streptomycin (Beyotime Inc., Shanghai, China) at 37°C in 5% CO₂ and 95% air atmosphere.

Reagents: Trizol Reagent (Invitrogen Inc., Grand Island, NY, USA); primers and probes (Invitrogen Inc., Grand Island, NY, USA); SYBR Green I;

SuperScript III Reverse Transcriptase; DEPC H₂O (Invitrogen Inc., Grand Island, NY, USA); Rnase inhibitor (Fermentas Inc., Hanover, MD, USA); 100mM dNTPs (Invitrogen Inc., Grand Island, NY, USA); oligo dT/primer; Platinum Taq DNA Polymerase; anti-NPY1R antibody, anti-estrogen receptor antibody, anti-progesterone receptor antibody, secondary antibody (Abcam Inc., Cambridge, MA, USA); phosphate-buffered solution (PBS); 0.05% Trypsin (Invitrogen Inc., Waltham, MA, USA); Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies Inc., Rockville, MD, USA); Transwell plates (8 µm; Corning Inc., New York, USA); crystal violet; [Leu31, Pro34]-Neuropeptide Y (human); BIBO-3304 (Sigma-Aldrich Inc., St. Louis, MI, USA).

Immunohistochemistry (IHC)

Uterine leiomyosarcoma tissue and normal tissue surrounding tumor samples from patients with uterine leiomyosarcoma were utilized for experiments. The research study was approved by institutional research board (IRB) of Ningbo First Hospital, and consent from patients was obtained. Tissue samples were embedded and sliced. Tissue samples were fixed in 4% paraformaldehyde, and blocked with bovine serum albumin for 30 min. The tumor and normal tissue samples were then incubated with primary antibodies of NPY1R, estrogen receptor, progesterone receptor (1:100) at 4°C overnight, respectively. After overnight incubation, cover slips were washed with PBS, and incubated in dark with goat anti-rabbit secondary antibody (1:1000) at room temperature for 1 h. Cover slides were washed with PBS. Slides were prepared with an anti-quenching mounting medium, and were observed with fluorescence microscope.

Quantitative polymerase chain reaction (qPCR)

SK-UT-1 cells were treated with estradiol (10 nM, 100 nM, 1 µM and 10 µM), and progesterone (10 nM, 100 nM, 1 µM and 2 µM) for 24 h, respectively. Relative expression of NPY1R after treatment of estradiol or progesterone was detected by qPCR. Total RNA was extracted and purified by Trizol respectively following manufacturer's instructions. A universal cDNA synthesis kit was utilized for reverse transcription. Each reaction contained 0.5 µL of random primers (0.2 µg/µL) and 1 µL of SuperScript III reverse transcriptase (200 U/µL). The specific

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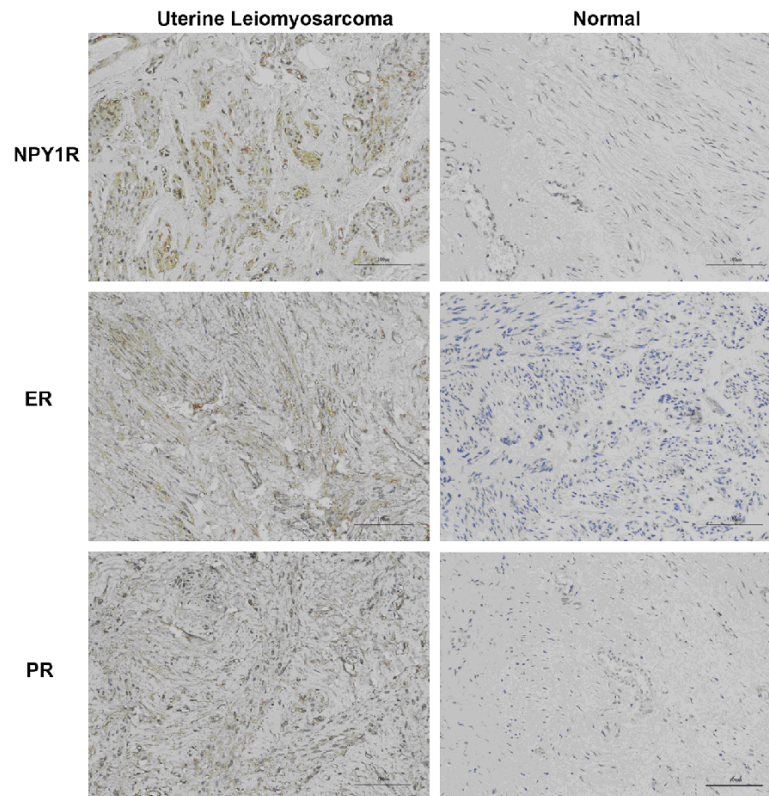


Figure 1. NPY1R was expressed in human uterine leiomyosarcoma tissue (200 \times). Uterine leiomyosarcoma tissue and normal tissue surrounding tumors from patients with uterine leiomyosarcoma were stained with antibodies of NPY1R, estrogen receptor, and progesterone receptor during immunohistochemistry staining. Similar to estrogen and progesterone receptors, we demonstrated that NPY1R was expressed in uterine leiomyosarcoma tissue, but not in normal tissue surrounding tumors. NPY1R: Neuropeptide Y Receptor Y1. ER: estrogen receptor. PR: progesterone receptor. Scale bar: 100 μ m.

primer for NPY1R was F: CCACTCTCCTCTTG-GTGCTG; R: GAGCCAGCAGACTGCAAATG. The primer for matrix metalloproteinase (MMP) 2 was F: GATACCCCTTTGACGGTAAGGA; R: CC-TTCTCCCAAGGTCCATAGC. The primer for MMP9 was F: TGTACCGCTATGGTTACTACTCG; R: GGCA-GGGACAGTTGCTTCT. qPCR was performed by utilizing MiRcute miRNA qPCR Detection kit. PCR conditions were: pre-denaturing at 95 $^{\circ}$ C for 2 min; denaturing at 95 $^{\circ}$ C for 10 s; and annealing and polymerization at 60 $^{\circ}$ C for 30 s, and 70 $^{\circ}$ C for 45 s. There were 40 PCR cycles. PCR was performed in CFX96 TouchTM Real-Time PCR Detection System. The gene expression was determined as the ratio of relative optical density of target gene to β -actin. $\Delta\Delta$ Ct method was utilized to measure PCR results.

Cell viability assay

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100

nM, 1 μ M and 2 μ M) for 24 and 48 h, respectively. In addition, SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μ M) for 24 and 48 h. The viability of SK-UT-1 cells was measured by CCK-8 cell viability assay kit. CCK-8 reagent was added into each well and incubated for 4 h. The absorbance was measured utilizing a microplate reader at 490 nm. Relative tumor cell viability rate was calculated by dividing the reading of each group at 24 h or 48 h by baseline reading at 0 h, respectively. Experiments were repeated for 3 times.

Cell proliferation assay

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μ M and 10 μ M) for 24 h, respectively. Cell proliferation rates were detected by 5-bromo-2'-deoxyuridine (BrdU) method. Cells were incubated with BrdU (10 μ M) for 1 h. Cells

were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 20 min. Cells were then treated with 0.25% TRITONTM X-100/PBS at room temperature for 20 min, and 2 M HCl at 37 $^{\circ}$ C for 30 min. Cells were incubated with primary antibody at 4 $^{\circ}$ C overnight, and secondary antibody at 37 $^{\circ}$ C for 45 min. Cells were then stained with DAPI (1:10000) at room temperature for 10 min. Cells were observed under fluorescent microscope.

Transwell invasion assay

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μ M and 10 μ M) for 48 h, respectively. In addition, SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μ M) for 48 h. The membrane of the upper compartment was coated with Matrigel (1 g/L; 50 μ L), and incubated at 37 $^{\circ}$ C for 1 h. Cell suspen-

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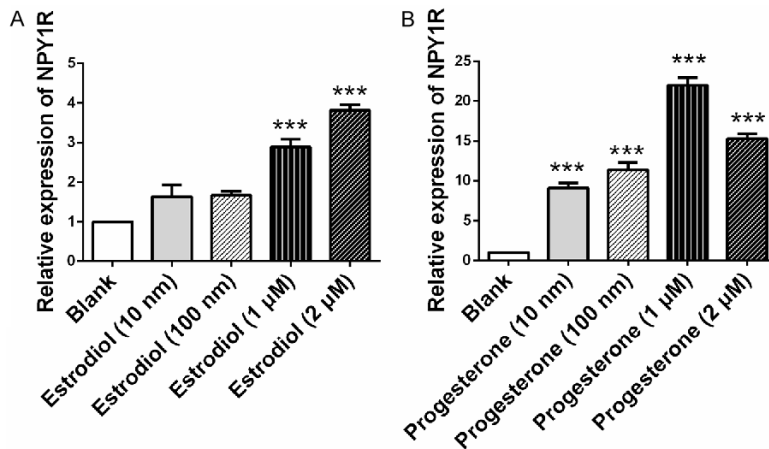


Figure 2. Estradiol and progesterone treatment significantly increased NPY1R expression in human uterine leiomyosarcoma SK-UT-1 cells. A. Effect of estradiol treatment on NPY1R expression; B. Effect of progesterone treatment on NPY1R expression. Human uterine leiomyosarcoma SK-UT-1 cells were treated with estradiol (10 nM, 100 nM, 1 μM and 2 μM), and progesterone (10 nM, 100 nM, 1 μM and 2 μM) for 24 h, respectively. Expression of NPY1R after treatment of estradiol or progesterone was detected by qPCR. Relative expression of NPY1R in experimental groups compared to blank group was presented in figures. Compared to non-treated SK-UT-1 cells, estradiol treatment at higher dosages (1 and 2 μM) markedly increased NPY1R expression. In addition, progesterone treatment also significantly increased NPY1R expression at all dosages used in current experiments (mean ± SD, n = 3/group). ***indicates that *p*-value < 0.001 when compared to blank group. NPY1R: Neuropeptide Y Receptor Y1.

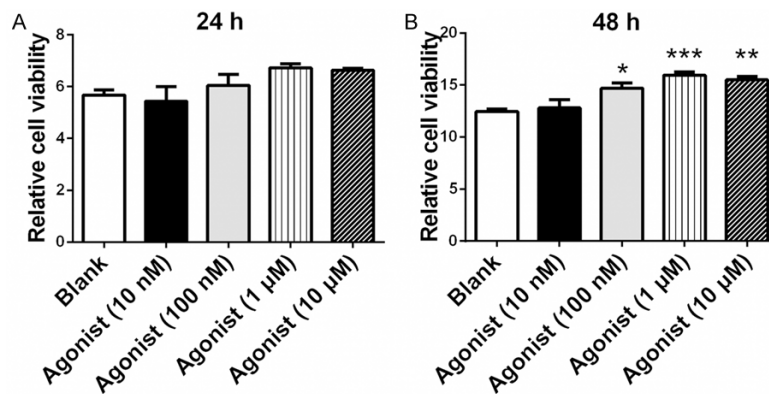


Figure 3. NPY1R agonist significantly increased the viability of SK-UT-1 cells at higher dosages at 48 h. SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μM and 10 μM) for 24 and 48 h, respectively. The viability of SK-UT-1 cells was measured by CCK-8 cell viability assay kit. Relative tumor cell viability rate was calculated by dividing the reading of each group at 24 h or 48 h by baseline reading at 0 h, respectively. The relative viability rates were not statistically different among groups at 24 h. However, NPY1R agonist significantly increased viability rate of SK-UT-1 cells at higher dosages (100 nM, 1 μM and 10 μM) at 48 h (mean ± SD, n = 3/group). *, ** and *** indicate that *p*-value < 0.05, 0.01, and 0.001 respectively when compared to blank group.

sion (200 μL) was incubated in upper compartment of Transwell, and 800 μL of culture medi-

um containing fetal bovine serum (FBS) was added into lower compartment. Cells were incubated at a humid incubator with 5% CO₂ for 24 h. Following culture, 4% paraformaldehyde was utilized to fix microporous membrane for 30 min. Cells were stained with 1% crystal violet for 10 min, and washed with PBS twice. Cells were then observed under microscope (400×), and the number of cells that penetrated the membrane was counted. Relative tumor cell invasion was calculated by dividing the average number of cells that penetrated membrane in the experimental groups by the average number of cells that penetrated membrane in the blank group. Experiments were repeated for 3 times.

Statistical analysis

The statistical data were analyzed by GraphPad Prism 5.0 software (GraphPad Software Inc.; La Jolla, CA, USA). Statistical results are demonstrated as mean ± SD. Differences among 3 or more groups were compared by analysis of variance (ANOVA), followed by the Bonferroni post-hoc test for multiple comparisons. *p* ≤ 0.05 was considered statistically significant.

Results

NPY1R was expressed in human uterine leiomyosarcoma tissue

Uterine leiomyosarcoma tissue and normal tissue surrounding tumors from patients with uterine leiomyosarcoma were stained with antibodies of NPY1R, estrogen recep-

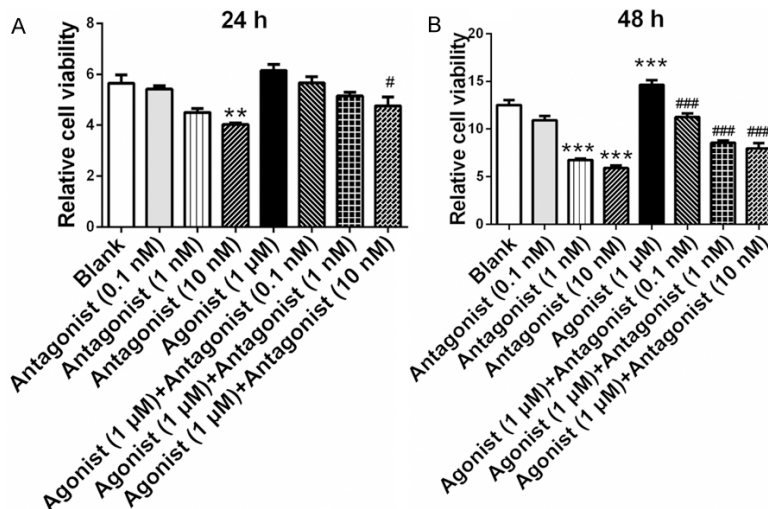


Figure 4. NPY1R antagonist markedly decreased the viability of SK-UT-1 cells at higher dosages. SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μM) for 24 and 48 h. The viability of SK-UT-1 cells was measured by CCK-8 cell viability assay kit. Compared to blank group, NPY1R antagonist (10 nM) dramatically decreased viability rate of SK-UT-1 cells at 24 h. Meanwhile, in presence of NPY1R agonist, NPY1R antagonist (10 nM) also significantly decreased viability rate of SK-UT-1 cells at 24 h, compared to NPY1R agonist alone group. In addition, the viability inhibition of SK-UT-1 cells by NPY1R antagonist was more prominent at 48 h. Compared to blank group, NPY1R antagonist (1 and 10 nM) dramatically decreased viability rate of SK-UT-1 cells at 48 h. Moreover, NPY1R agonist (1 μM) markedly increased viability rate of SK-UT-1 cells at 48 h, compared to blank group. NPY1R antagonist (0.1, 1 and 10 nM) significantly decreased the viability rate of SK-UT-1 cells at 48 h, compared to NPY1R agonist alone group (mean ± SD, n = 3/group). ** and *** indicate that p -value <0.01 and 0.001 respectively when compared to blank group. # and ### represent that p -value <0.05 and 0.001 respectively when compared to NPY1R agonist group.

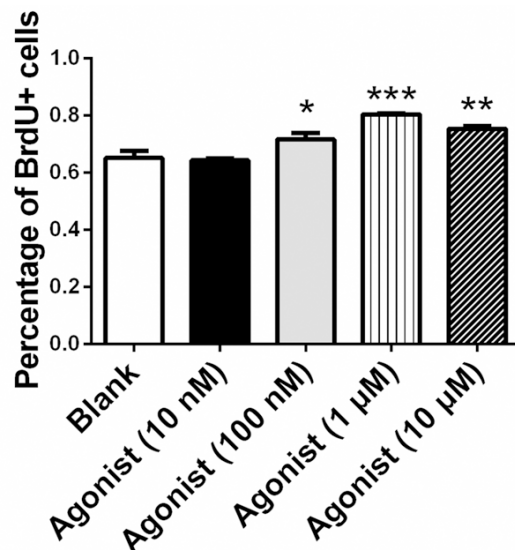


Figure 5. NPY1R agonist significantly increased the proliferation of SK-UT-1 cells at higher dosages at 24 h. SK-UT-1 cells were treated with NPY1R agonist

[Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μM and 10 μM) for 24 h, respectively. The proliferation of SK-UT-1 cells was measured by BrdU method. NPY1R agonist significantly increased the percentages of BrdU+ cells at higher dosages (100 nM, 1 μM and 10 μM) at 24 h (mean ± SD, n = 3/group). *, ** and *** indicate that p -value <0.05, 0.01 and 0.001 respectively when compared to blank group.

tor, and progesterone receptor during immunohistochemistry staining. Similar to estrogen and progesterone receptors, we demonstrated that NPY1R was expressed in uterine leiomyosarcoma tissue, but not in normal tissue surrounding tumors (Figure 1).

Estrodiol and progesterone treatment significantly increased NPY1R expression in human uterine leiomyosarcoma SK-UT-1 cells

Human uterine leiomyosarcoma SK-UT-1 cells were treated with estradiol (10 nM, 100 nM, 1 μM and 2

μM), and progesterone (10 nM, 100 nM, 1 μM and 2 μM) for 24 h, respectively. Expression of NPY1R after treatment of estradiol or progesterone was detected by qPCR. Relative expression of NPY1R in experimental groups compared to blank group was presented in figures. Compared to non-treated SK-UT-1 cells, estradiol treatment at higher dosages (1 and 2 μM) markedly increased NPY1R expression (p < 0.001, Figure 2A). In addition, progesterone treatment also significantly increased NPY1R expression at all dosages used in current experiments (10 nM, 100 nM, 1 μM and 2 μM; p < 0.001, Figure 2A).

NPY1R agonist significantly increased the viability of SK-UT-1 cells at higher dosages at 48 h

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100

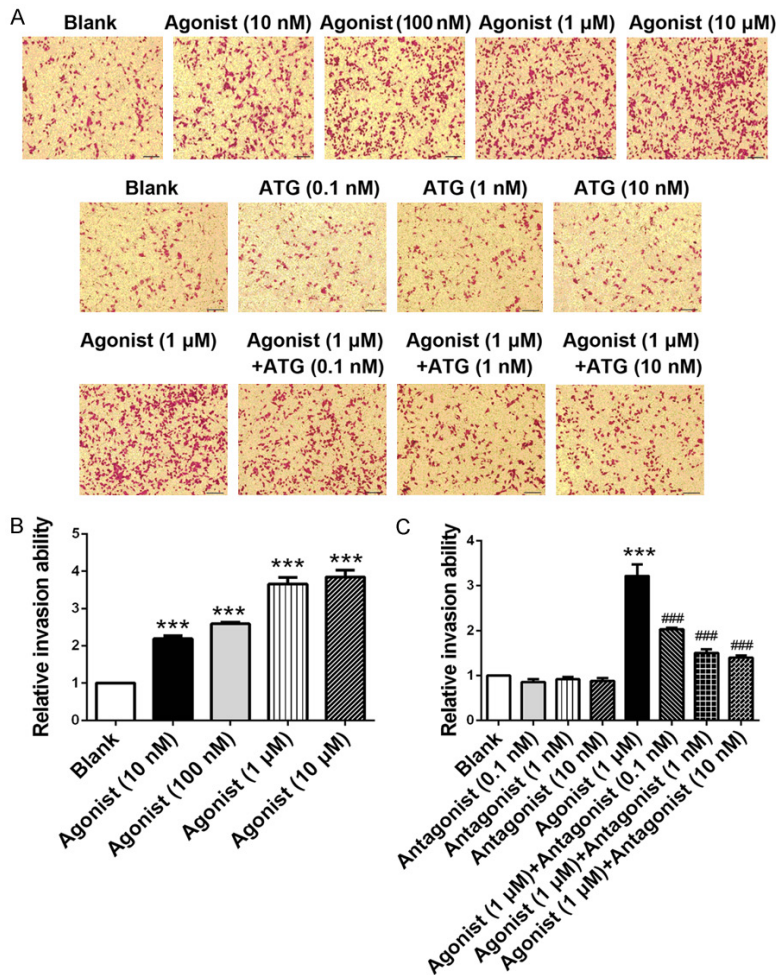


Figure 6. NPY1R agonist markedly increased the invasion ability of SK-UT-1 cells dose-dependently, and NPY1R antagonist significantly decreased the invasion ability of SK-UT-1 cells in presence of NPY1R agonist. A. Representative results of transwell invasion assay (100×). SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μM and 10 μM) for 48 h, respectively. In addition, SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μM) for 48 h. Transwell invasion assay was utilized to detect tumor invasion ability. Cells were then observed under microscope, and the number of cells that penetrated the membrane was counted. B. Relative invasion ability of SK-UT-1 cells treated with NPY1R agonist. C. Relative invasion ability of SK-UT-1 cells treated with NPY1R agonist and antagonist. Relative tumor cell invasion was calculated by dividing the average number of cells that penetrated membrane in experimental groups by the average number of cells that penetrated membrane in the blank group. Compared to blank group, NPY1R agonist markedly increased the invasion ability of SK-UT-1 cells dose-dependently. NPY1R antagonist did not alter the invasion ability of SK-UT-1 cells compared to blank group. However, in presence of NPY1R agonist, NPY1R antagonist significantly decreased the invasion ability of SK-UT-1 cells, as compared to NPY1R agonist alone group (mean ± SD, n = 3/group). ***indicates that p -value < 0.001 when compared to blank group. ###represents that p -value < 0.001 when compared to NPY1R agonist group. ATG: antagonist.

nM, 1 μM and 10 μM) for 24 and 48 h, respectively. The viability of SK-UT-1 cells was mea-

sured by CCK-8 cell viability assay kit. Relative tumor cell viability rate was calculated by dividing the reading of each group at 24 h or 48 h by baseline reading at 0 h, respectively. The relative viability rates were not statistically different among groups at 24 h. However, NPY1R agonist significantly increased viability rate of SK-UT-1 cells at higher dosages (100 nM, 1 μM and 10 μM) at 48 h (p <0.05, **Figure 3B**).

NPY1R antagonist markedly decreased the viability of SK-UT-1 cells at higher dosages

SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μM) for 24 and 48 h. The viability of SK-UT-1 cells was measured by CCK-8 cell viability assay kit. Compared to blank group, NPY1R antagonist (10 nM) dramatically decreased viability rate of SK-UT-1 cells at 24 h (p <0.01, **Figure 4A**). Meanwhile, in presence of NPY1R agonist, NPY1R antagonist (10 nM) also significantly decreased viability rate of SK-UT-1 cells at 24 h, compared to NPY1R agonist alone group (p <0.05, **Figure 4A**). In addition, the viability inhibition of SK-UT-1 cells by NPY1R antagonist was more prominent at 48 h. Compared to blank group, NPY1R antagonist (1 and 10 nM) dramatically decreased viability rate of SK-UT-1 cells at 48 h (p <0.001, **Figure 4B**). Moreover, NPY1R agonist (1 μM) markedly increased viability rate of SK-UT-1 cells at 48 h, compared to blank group (p <0.001, **Figure 4B**). NPY1R antagonist

rate of SK-UT-1 cells at 48 h, compared to blank group (p <0.001, **Figure 4B**). NPY1R antagonist

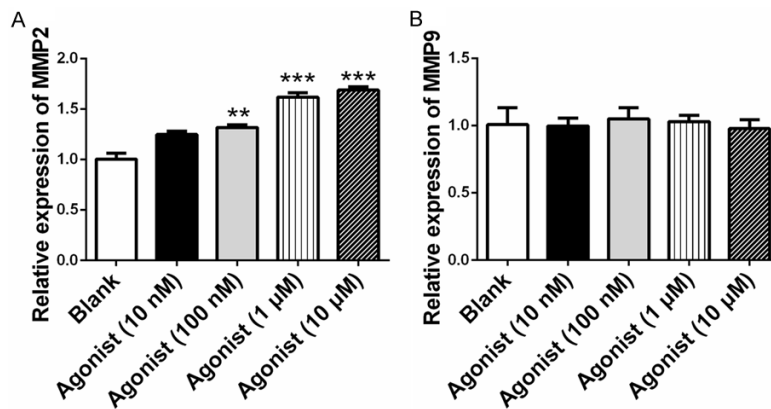


Figure 7. NPY1R agonist significantly increased the expression of MMP2 in SK-UT-1 cells at higher dosages at 48 h. The expression of MMP2 and MMP9 was evaluated by qPCR. Compared to blank group, NPY1R agonist significantly increased the expression of MMP2 in SK-UT-1 cells dose-dependently. NPY1R agonist did not alter the expression of MMP9 in SK-UT-1 cells (mean \pm SD, $n = 3$ /group). *, ** and *** indicate that p -value < 0.05 , 0.01 and 0.001 respectively when compared to blank group. MMP: matrix metalloproteinases.

(0.1, 1 and 10 nM) significantly decreased the viability rate of SK-UT-1 cells at 48 h, compared to NPY1R agonist alone group ($p < 0.001$, **Figure 4B**).

NPY1R agonist significantly increased the proliferation of SK-UT-1 cells at higher dosages at 24 h

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μ M and 10 μ M) for 24 h, respectively. The proliferation of SK-UT-1 cells was measured by BrdU method. NPY1R agonist significantly increased the percentages of BrdU+ cells at higher dosages (100 nM, 1 μ M and 10 μ M) at 24 h ($p < 0.05$, **Figure 5**).

NPY1R agonist markedly increased the invasion ability of SK-UT-1 cells dose-dependently, and NPY1R antagonist significantly decreased the invasion ability of SK-UT-1 cells in presence of NPY1R agonist

SK-UT-1 cells were treated with NPY1R agonist [Leu31, Pro34]-Neuropeptide Y (10 nM, 100 nM, 1 μ M and 10 μ M) for 48 h, respectively. In addition, SK-UT-1 cells were treated with NPY1R antagonist BIBO-3304 (0.1, 1, 10 nM) in presence or absence of NPY1R agonist (1 μ M) for 48 h. Transwell invasion assay was utilized to detect tumor invasion ability. Relative tumor cell invasion was calculated by dividing the

average number of cells that penetrated membrane in experimental groups by the average number of cells that penetrated membrane in the blank group. Compared to blank group, NPY1R agonist markedly increased the invasion ability of SK-UT-1 cells dose-dependently ($p < 0.001$, **Figure 6A** and **6B**). NPY1R antagonist did not alter the invasion ability of SK-UT-1 cells compared to blank group. However, in presence of NPY1R agonist, NPY1R antagonist significantly decreased the invasion ability of SK-UT-1 cells, as compared to NPY1R agonist alone group ($p < 0.001$, **Figure 6A** and **6C**).

In addition, the expression of MMP2 and MMP9 was evaluated by qPCR at 48 h. Compared to blank group, NPY1R agonist significantly increased the expression of MMP2 in SK-UT-1 cells dose-dependently ($p < 0.001$, **Figure 7A**). NPY1R agonist did not alter the expression of MMP9 in SK-UT-1 cells.

Discussions

We have demonstrated in current study that NPY1R was expressed in human uterine leiomyosarcoma tissue. Estradiol and progesterone can increase NPY1R expression. NPY1R agonist significantly increased proliferation and invasion of human uterine leiomyosarcoma cells, and NPY1R antagonist markedly decreased the proliferation and invasion, especially in presence of NPY1R agonist.

Neuropeptide Y was reported to inhibit the trigeminovascular pathway through NPY1R, and was implicated in migraine [10]. The interactions between galanin receptor 2 and NPY1R in the dentate gyrus were revealed to be associated with antidepressant-like effects [11]. Meanwhile, adult exposure to tributyltin affected the distribution of hypothalamic NPY1R, and circulating leptin in mice [12]. Neuropeptide Y also induced hematopoietic stem/progenitor cell mobilization by regulating matrix metalloproteinase-9 activity via NPY1R in osteoblasts [13]. Moreover, NPY1R regulated glucocorti-

coid-induced inhibition of osteoblast differentiation in murine MC3T3-E1 cells via ERK signaling [14]. In current study, we demonstrated that NPY1R was expressed in human uterine leiomyosarcoma tissue, but not in normal tissue surrounding tumors. This broadens current understanding of role of NPY1R in various diseases, and shed light on possible pharmacological treatment targeting NPY1R.

Estrogen and progesterone receptors are frequently expressed in uterine leiomyosarcoma [15, 16]. Expression of progesterone receptor was associated with disease-free survival but not overall survival of uterine leiomyosarcoma [17]. High levels of NPY1R expression were reported to be positively correlated with the status of estrogen and progesterone receptors in breast cancer tissue [9]. In current study, we demonstrated that NPY1R was expressed in uterine leiomyosarcoma tissue, together with expression of estrogen and progesterone receptors.

In addition, we revealed that estradiol and progesterone can increase NPY1R expression in human uterine leiomyosarcoma cells. Deletion of estrogen-related receptor beta correlated with attenuated expression of neuropeptide Y in hindbrain [18]. Estrogen was shown to facilitate both phosphatidylinositol 3-kinase/Akt and ERK1/2 mitogen-activated protein kinase membrane signaling required for long-term neuropeptide Y transcriptional regulation in clonal immortalized neurons [19]. Moreover, estradiol stimulated gene transcription of mouse NPY1R by binding to estrogen receptor alpha in neuroblastoma cells [20]. Estrogen also up-regulated expression of NPY1R in a human breast cancer cell line [21]. In addition, enhanced food intake by progesterone-treated female rats was related to changes in neuropeptide genes expression in hypothalamus [22]. Expression of NPY1R gene was also reported to be increased in the medial amygdala of transgenic mice after long-term treatment with progesterone [23]. The detailed molecular mechanism underlying the increased expression of NPY1R in uterine leiomyosarcoma cells stimulated by estrogen and progesterone require further research investigations.

Studies unveiling the relationship between NPY1R and tumor progression are scarce.

Breast cancer patients with circulating cancer cells that expressed NPY1R were reported to exhibit shorter tumor-specific survival when compared with those with no NPY1R expression [9]. In current study, we demonstrated that NPY1R agonist significantly increased proliferation and invasion of human uterine leiomyosarcoma cells, and NPY1R antagonist markedly decreased the proliferation and invasion, especially in presence of NPY1R agonist. These indicate the positive correlation between activation of NPY1R and tumor progression of uterine leiomyosarcoma. Therefore, targeting NPY1R may provide better prognosis in patients with uterine leiomyosarcoma. More research investigation, such as animal model studies, are needed to elucidate effects of NPY1R blockade on long-term survival in uterine leiomyosarcoma.

In conclusion, this study highlights the crucial role of NPY1R in promoting the proliferation and invasion of human uterine leiomyosarcoma cells. Although more studies are needed to elucidate detailed molecular pathways involving NPY1R, these results pave the foundation for possible pharmaceutical targeting of uterine leiomyosarcoma.

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Disclosure of conflict of interest

None.

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