Neurokinin-1 receptor is highly expressed in cervical cancer and its antagonist induces cervical cancer cell apoptosis

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ABSTRACT

Neurokinin-1 receptor (NK1R) belongs to tachykinin receptor family. Recent studies have suggested that NK1R was upregulated in cancer tissues including breast cancer, glioma and melanoma. Furthermore, NK1R antagonists have been employed to exert anti-tumor effect and promote cancer cell apoptosis. However, the role of NK1R in cervical cancer remains largely unknown. In this study, we aimed to detect the expression of NK1R in cervical cancer and evaluate the anti-tumor effects of NK1R antagonist on cervical cancer cells. We found that NK1R was highly expressed in cervical cancer tissues than in adjacent normal cervical tissues. Furthermore, by using NK1R antagonist we demonstrated that NK1R antagonist inhibited the viability and induced the apoptosis of cervical cancer cells in a dose-dependent manner, and the mechanism may be related to the inhibition of ERK activation and the regulation of apoptosis proteins Bcl-2 and BAX. In conclusion, these findings suggest that NK1R plays an oncogenic role in cervical cancer and is a promising target for cervical cancer therapy.

Key words: xNeurokinin-1 receptor; ERK; cervical cancer; apoptosis.

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Contributions: HX, designed the study; QY, HX, performed all experiments. All the authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

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Introduction

Cervical cancer is one common malignant cancer in the women worldwide. While the pathogenesis of cervical cancer involves multiple genetic and environmental factors, it is considered that the infection with human papillomavirus is a possible cause of cervical cancer.[1,2] For cervical cancer patients at early stage, timely diagnosis and treatment have significantly improved their 5-year survival. However, for cervical cancer patients at advanced stage, their 5-year survival is still very poor despite comprehensive treatment strategies such as the combined radiotherapy and chemotherapy. Therefore, current efforts focus on deeper understanding of the molecular mechanism of cervical cancer in order to identify innovative therapeutic targets.[3]

G protein-coupled receptor (GPCR) is a group of the largest superfamily of cell membrane proteins, and plays an important role in the initiation and development of various types of cancers. [4,5] Neurokinin-1 receptor (NK1R) is a type of GPCR and belongs to tachykinin receptor family. Recent studies have suggested that NK1R may function as an oncogenic GPCR.[6] For example, NK1R was upregulated in cancer tissues including breast cancer, glioma and melanoma.[7,8] In addition, an endogenous ligand of NK1R called peptide substance P could inhibit chemotherapy-induced apoptosis of cancer cells by activating NK1R.[9,10] Furthermore, NK1R antagonists have been employed to exert anti-tumor effect and promote cancer cell apoptosis.[11,12] A recent study reported that NK1R could be a potential therapeutic target for cervical cancer by using *in vitro* cervical cancer cell model.[13] In this study, we aimed to detect the expression of NK1R in cervical cancer and evaluate the anti-tumor effects of NK1R antagonist on cervical cancer cells.

Materials and Methods

Tissue samples

This study was approved by the Ethical Review Committee of Hebei Medical University (approval No. 2018019). All patients provided written informed consent. Five formalin-fixed, paraffin-embedded cervical cancer samples were collected from 5 cervical cancer patients, while matched adjacent normal cervical tissues were collected as corresponding controls.

Immunohistochemical staining

Normal cervical tissues and cervical cancer tissues were fixed in 10% formalin and then embedded in paraffin. The tissues were cut into 5 µm serial sections and processed for immunohistochemical staining. Antigen retrieval was performed by incubation of the sections in 10 mM citrate buffer (pH 6.0) at 100°C, and the sections were treated with 3% hydrogen peroxide for 1 h to block endogenous peroxidases. Next, the sections were incubated with NK1R antibody (Abbott Diagnostics, Chicago, IL, USA; 1:500 dilution) at 37°C for 1 h, washed with phosphate buffered saline (PBS), and then incubated with secondary antibody (Roche Diagnostics, Indianapolis, IN, USA; 1:2,000 dilution) at 37°C for 1 h. The sections were washed with PBS and incubated with DAB detection kit (Roche Diagnostics) for visualization. All slides were evaluated by two pathologists independently. For negative controls, the sections were incubated with PBS instead of NK1R antibody. In each slide, high-power microscopic fields were evaluated at 40× magnification. The intensity of the immunostaining was scored as following: when less than 10% of the total cells were stained, the staining intensity was considered low (+1); when 10-40% of the total cells were stained, the staining intensity was considered moderate (+2); when more than 40% of the total cells were stained the staining intensity was considered high (+3).

Cell culture

Human cervical cancer cell line SiHa was purchased from American Type Culture Collection and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Thermo Fisher, Waltham, MA, USA) at 37°C with 5% CO2. NK1R antagonist L-733,060 was purchased from R&D Systems (Abingdon, UK).

MTT assay

SiHa cells were seeded in 96-well plates at 2 x 10^3 cells/plate and treated with 5 µM, 10 µM or 20 µM L-733,060 for 48 h. The cells were collected and evaluated with MTT assay kit (Sigma-Aldreich, St. Louis, MO, USA) according to the manufacturer's instructions. 20 µL MTT (5 mg/mL) solution was added to each well and the plates were incubated at 37˚C for 4 h, then 150 µL DMSO was added to each well and the plates were incubated at room temperature for 10 min. The absorption value of every well was read at 490 nm using a microplate reader (Sunrise model; Tecan, Männedorf, Switzerland) and was analyzed with Magellan Data Analysis Software (Tecan). All wells were in triplicates.

Flow cytometry

SiHa cells were seeded in 96-well plates at 2x10^3 cells/plate and treated with different concentrations of L-733,060 for 48 h. The cells were collected and the apoptosis was examined by Annexin V/FITC apoptosis kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's protocol. The stained cells were immediately analyzed using FACS Calibur System LSR II (BD Biosciences). About 1,000 cells were counted for each group and each group had three replicates. The percentage of positively stained cells was calculated to assess apoptosis ratio.

Western blot analysis

SiHa cells were collected, and total proteins were extracted using RIPA buffer. The protein concentration was determined by using bicinchoninic acid assay. Equal amounts of proteins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Herculs, CA, USA). The membranes were blocked in 5% non-fat milk and then incubated with primary antibodies for ERK, p-ERK, Bcl-2, BAX and GAPDH (1:500 dilution, all from Abcam, Cambridge, UK) at 4°C overnight. The membranes were washed and incubated with secondary antibodies (Abcam; 1:1,000 dilution) at room temperature for 1 h, and detected by chemiluminescence. Densitometry analysis of the bands was performed using Image-J software with GAPDH as loading control.

Statistical analysis

All data were presented as the mean± standard deviation (SD) and analyzed by SPSS statistical software (IBM, Chicago, IL, USA). Comparisons between groups were performed using Student's *t*-test. Difference was considered significant for $p<0.05$.

Results

NK1R expression was higher in cervical cancer tissues than in adjacent normal tissues

Immunohistochemical staining showed that NK1R was weakly stained in adjacent normal cervical tissues from patients with cervical cancer (Figure 1A). In contrast, NK1R was strongly stained

in cervical cancer tissues (Figure 1B). Among 5 patients with cervical cancer enrolled in this study, the staining intensity of NK1R was high for all 5 cervical cancer tissues (2 tissues had score of +2 and 3 tissues had score of +3) and was low for all 5 adjacent normal cervical tissues (all tissues had score of +1).

NK1R antagonist inhibited the viability of cervical cancer cells

To investigate the role of NK1R in cervical cancer, we treated cervical cancer SiHa cells with different concentrations of NK1R antagonist L-733,060. MTT assay showed that L-733,060 inhibited the viability of SiHa cells in a dose-dependent manner (Figure 2). These results indicate that NK1R may promote the viability of cervical cancer cells.

NK1R antagonist promoted the apoptosis of cervical cancer cells

To further examine the effects of NK1R antagonist on cervical cancer cell viability, we performed flow cytometry and found that L-733,060 enhanced the apoptosis of SiHa cells in a dose-dependent manner (Figure 3A). Quantitative analysis showed that apoptosis percentage was significantly higher in SiHa cells treated with higher concentrations of L-733,060 (Figure 3B). These results demonstrate that NK1R antagonist promoted the apoptosis of cervical cancer cells.

NK1R antagonist modulated the expression of apoptosis related molecules in cervical cancer cells

To investigate the mechanism by which NK1R antagonist promoted the apoptosis of cervical cancer cells, we detected the activation of ERK and the expression of apoptosis related proteins. Western blot analysis showed that L-733,060 inhibited the phosphorylation of ERK in SiHa cells in a dose-dependent manner, without affecting total ERK protein levels (Figure 4A). Moreover, L-733,060 inhibited the expression of anti-apoptotic protein Bcl-2 while increased the expression of pro-apoptotic protein BAX in SiHa cells in a dose-dependent manner (Figure 4A). Densitometry analysis showed that protein levels of p-ERK and Bcl-2 were significantly lower, while protein levels of BAX were significantly higher in SiHa cells treated with higher concentrations of L-733,060 (Figure 4B).

Discussion

In this study we reported that NK1R was highly expressed in cervical cancer tissues than in adjacent normal cervical tissues. Furthermore, by using NK1R antagonist we demonstrated that NK1R antagonist inhibited the viability and induced the apoptosis of cervical cancer cells, and the mechanism may be related to the

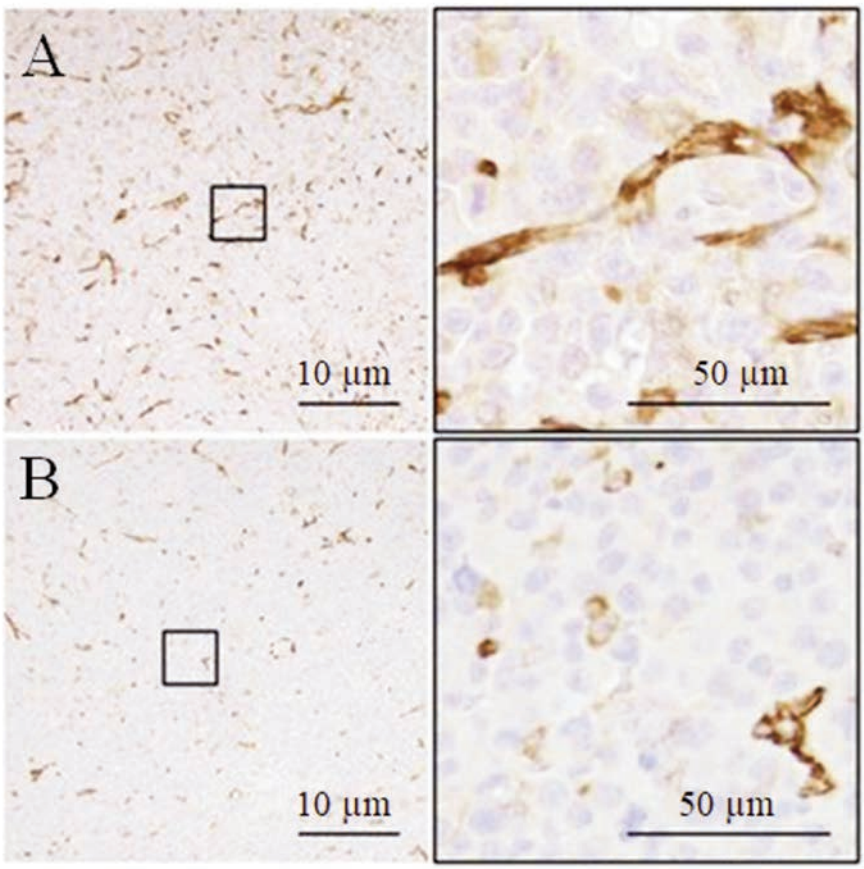

Figure 1. Immunohistochemical staining of NK1R in cervical cancer tissues. A. Representative staining of NK1R in cervical cancer tissues. The staining signal was very strong as shown by dark brown. B. Representative staining of NK1R in adjacent normal cervical tissues. The staining signal was very weak.

inhibition of ERK activation and the regulation of apoptosis proteins. In recent years, accumulating studies suggest that NK1R is highly expressed in a variety of tumor tissues and cells, and its expression is related to clinicopathological stage, lymph node metastasis and prognosis of cancer patients.[14-17] Zhang *et al*. found that the truncated NK-1 receptor (NK1R-Tr) but not the full-length NK-1 receptor (NK1R-FL) was highly expressed in breast cancer, and its expression was correlated with tumor grade and Ki-67.[18] Muñoz *et al.* used NK1R antagonist L-732,138 to treat colon cancer SW-403 cells and gastric cancer 23132-87 cells, and found that L-732,138 could significantly inhibit the proliferation and induce apoptosis of the two cell lines.[19] Nizam *et al*. found that NK1R antagonists significantly inhibited the liver metastasis of tumor cells in mice, and alleviated inflammatory stress response.[20] More recently, it was reported that NK1R antagonist inhibited HeLa cell viability.[13] Consistent with these previously reported results, in this study we found that NK1R antagonist significantly inhibited the proliferation and induced the apoptosis of SiHa cells, indicating that NK1R plays an oncogenic role in cervical cancer and is a potential therapeutic target.

Extracellular signal-regulated protein kinase (ERK) is a subfamily of MAPKs family. The phosphorylated p-ERK is activated and regulates the expression of various downstream genes by transmitting signals from the cytoplasm to the nucleus to regulate cell proliferation and differentiation.[21] The results of this study showed that NK1R antagonist can significantly inhibit the phosphorylation of ERK in cervical cancer cells, correlated with the inhibition of the proliferation of Siha cells. Bcl-2 is located on the mitochondrial membrane and is the most important gene for apoptosis. Studies have shown that Bcl-2 can inhibit apoptosis by interfering with the activity of Caspase-3, and can play an anti-cellular role by maintaining mitochondrial membrane potential and controlling reactive oxygen species.[22] In contrast, BAX is an important protein that promotes apoptosis. The results of this study confirmed that NK1R antagonist downregulated the expression of Bcl-2 and upregulated the expression of BAX in SiHa cells, indicating that NK1R antagonist can promote cervical cancer cell apoptosis.

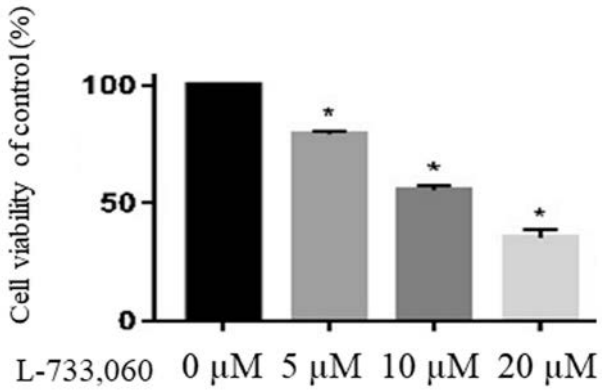

Figure 2. NK1R antagonist inhibited the viability of cervical cancer cells. SiHa cells were treated with different concentrations of NK1R antagonist L-733,060 (from 0 to 20 μM). MTT assay showed that L-733,060 inhibited the viability of SiHa cells in a dose-dependent manner. Data were expressed as the mean ± SD (n=3). *p<0.05 compared to control cells not treated with L-733,060.

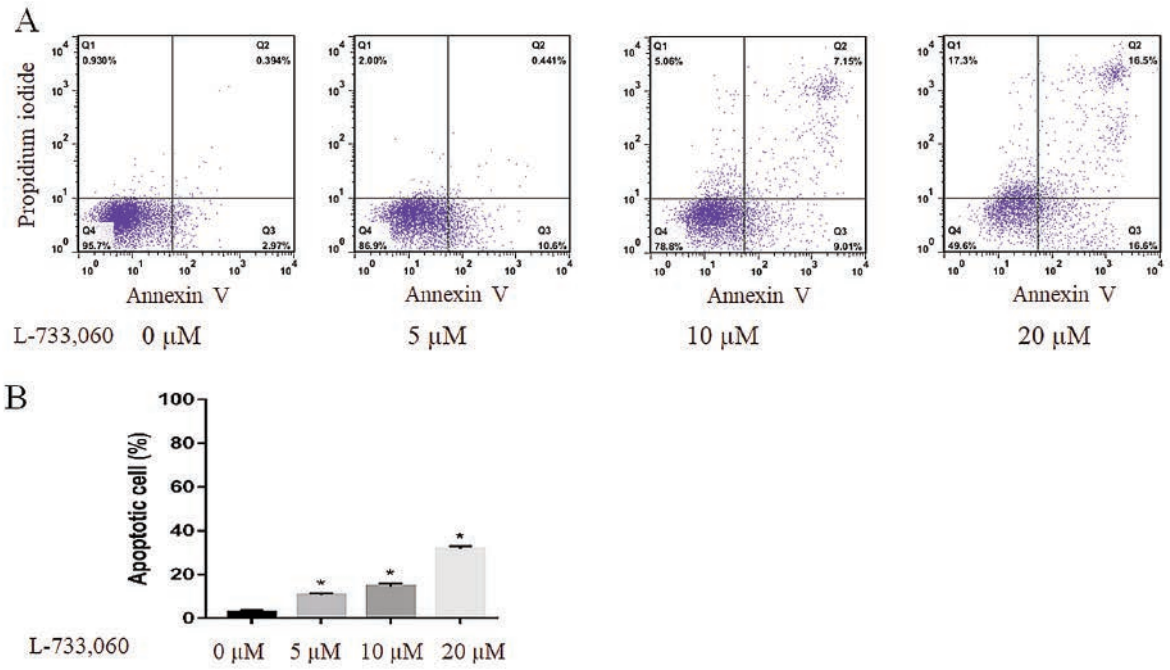

Figure 3. NK1R antagonist induced the apoptosis of cervical cancer cells. A) Flow cytometry analysis of apoptosis in SiHa cells treated with different concentrations of NK1R antagonist L-733,060 (from 0 to 20 μM). B) Quantitative analysis of apoptosis percentage in SiHa cells treated with different concentrations of NK1R antagonist L-733,060 (from 0 to 20 μM). Data were expressed as the mean ± SD (n=3). *p<0.05 compared to control cells not treated with L-733,060.

Further studies are needed to reveal the mechanism by which NK1R regulates ERK and apoptosis pathway.

Despite the study's main limitation, the number of patients examined, this study provides evidence that NK1R was highly expressed in cervical cancer tissues and that NK1R antagonist inhibited viability and induced apoptosis of cervical cancer cells, at least partially *via* inhibition of ERK activation and regulation of apoptosis proteins. These findings suggest that NK1R plays an oncogenic role in cervical cancer and represents a promising target for cervical cancer therapy.

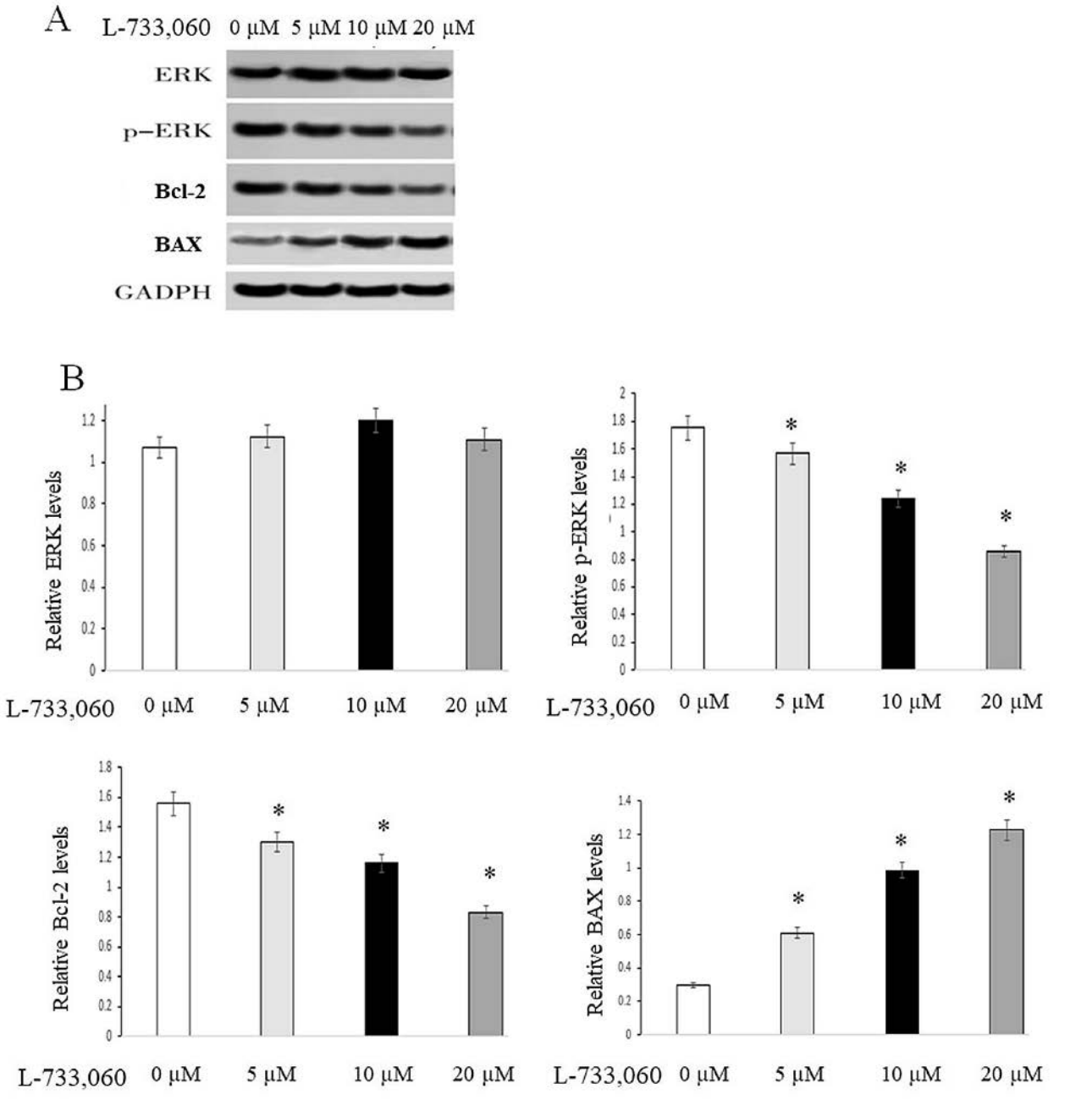

Figure 4. NK1R antagonist modulated the expression of apoptosis related molecules in cervical cancer cells. A) Western blot analysis of ERK, p-ERK, Bcl-2 and BAX protein levels in SiHa cells treated with different concentrations of NK1R antagonist L-733,060 (from 0 to 20 μM). GAPDH was loading control. B) Densitometry analysis of ERK, p-ERK, Bcl-2 and BAX protein levels in SiHa cells treated with different concentrations of NK1R antagonist L-733,060 (from 0 to 20 μM). Data were expressed as the mean ± SD (n=3). *p<0.05 compared to control cells not treated with L-733,060.

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