CANCER CACHEXIA is a multifactorial paraneoplastic syndrome consisting of ongoing skeletal muscle wasting and body weight reduction [1]. Most patients with advanced cancers suffer from difficulties related to cancer cachexia. The cachectic condition cannot be fully reversed by nutritional support alone. Ghrelin, a 28–amino-acid peptide, was originally isolated from human and rat stomach as an endogenous ligand of growth hormone secretagogue receptor (GHSR) [2]. The pleiotropic effects of ghrelin, including stimulation of food intake independently of growth hormone (GH) secretion, reduction of energy expenditure, stimulation of adiposity, and prevention of muscle wasting, suggest that it could be used to treat cachexia [3].

Ghrelin might also influence cancer progression, dependent on or independent of its effects on GHSR and insulin-like growth factor 1 (IGF-1) signaling [4]. Some studies reported that ghrelin plays a role in mechanisms related to cancer progression — including cell proliferation, invasion and migration, and resistance to apoptosis in cell lines from pancreatic, breast, and colorectal cancer [4]. By contrast, several in vitro studies have indicated that ghrelin treatment inhibits proliferation in cell lines from ovarian cancer, thyroid carcinoma, or prostate cancer [5]. The influence of ghrelin on lung cancer progression remains controversial, and ghrelin-mediated effects on lung cancer have not been elucidated. Cancer cachexia is closely linked to lung cancer [6], thus it is important to determine whether ghrelin promotes or inhibits cancer progression in this disease.

We here investigated the influence of ghrelin on several mechanisms related to cancer progression: cell viability, proliferation, resistance to apoptosis, and mitochondrial function. We used HLC-1 cells, a human lung adenocarcinoma cell line harboring KRAS mutation [7].

Ghrelin does not influence cancer progression in a lung adenocarcinoma cell line

Hironobu Tsubouchi1), Hitomi Onomura1), Yusuke Saito2), Shigehisa Yanagi1), Ayako Miura1), Ayako Matsuo1), Nobuhiro Matsumoto1) and Masamitsu Nakazato1), 3)

1) Division of Neurology, Respirology, Endocrinology and Metabolism, Department of Internal Medicine, University of Miyazaki, Miyazaki, Japan
2) Division of Pediatrics, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
3) Research director of CREST, Japan Agency for Medical Research and Development (AMED)

Abstract. Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (GHSR), is produced in the human stomach. Although ghrelin has therapeutic potential for cancer cachexia, ghrelin treatment may have a concern about accelerating cancer progression. Here, using the human lung adenocarcinoma cell line HLC-1, we investigated the effects of ghrelin on molecular mechanisms linked to cancer progression, including cell viability, proliferation, resistance to apoptosis, and mitochondrial activity. Both types of mouse alveolar epithelial cells (types I and II) expressed the GHSR, as did the human normal airway cell lines BEAS-2B and HLC-1. Treatment with ghrelin (10−2, 10−1, 1, 10 μM) did not affect cell viability or proliferation. Pretreatment of HLC-1 cells with ghrelin (10 μM) did not affect resistance to paclitaxel-induced apoptosis. The parameters of mitochondrial respiration, including basal respiration, proton leak, ATP production, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration, of the HLC-1 cells pretreated with or without ghrelin were unchanged. Taken together, ghrelin does not influence cancer progression in lung adenocarcinoma cells.

Key words: Ghrelin, Cancer progression, Lung adenocarcinoma, HLC-1, GHSR

Correspondence to: Hironobu Tsubouchi, M.D., Ph.D., Division of Neurology, Respirology, Endocrinology and Metabolism, Department of Internal Medicine, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan.
E-mail: hironobu_tsubouchi@med.miyazaki-u.ac.jp
©The Japan Endocrine Society
Materials and Methods

Animals
Ghsr-enhanced green fluorescent protein (eGFP) reporter mice were obtained from the Mouse Mutant Regional Resource Center at UC-Davis. Ten-week-old male mice were used for immunostaining of green fluorescent protein (GFP) in the lung. Mice were housed in a temperature-controlled room (23 ± 1°C) on a 12-hr light (08:00–20:00 h)/12-hr dark cycle and fed standard laboratory chow with ad libitum access to food. All experimental procedures were performed in accordance with the Japanese Physiological Society’s guidelines for animal care and were approved by the Ethics Committee on Animal Experimentation of the University of Miyazaki.

Chemicals
Paclitaxel (PTX) and human ghrelin were obtained from Sigma-Aldrich Japan (Tokyo, Japan) and Peptide Institute (Osaka, Japan), respectively, and dissolved in sterile PBS.

Cell lines
Human normal airway BEAS-2B cells (American Type Culture Collection, Rockville, MD) and human lung adenocarcinoma HLC-1 cells (RIKEN Cell Bank, Tsukuba Science City, Japan) were cultured in DMEM and Ham’s F12 media, respectively. Complete media were supplemented with fetal bovine serum, 50 U/mL penicillin, and 50 µg/mL streptomycin.

Immunostaining
For immunohistochemical analyses, we used Ghsr-eGFP reporter mice (n = 5). Mice were anesthetized by intraperitoneal injection of pentobarbital sodium. After sacrificing, the lungs were fixed in Amsterdam’s fixative (methanol : acetone : acetic acid : water, 35 : 35 : 5 : 25) and embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan). Lung sections (4 µm thick) were mounted on slides for immunostaining with antibody against surfactant protein-C (SP-C) (Santa Cruz Biotechnology, Dallas, TX, USA) or GFP (Medical & Biological Laboratories, Nagoya, Japan).

RT-PCR
Quantitative real-time polymerase chain reaction (RT-PCR) was performed using TaqMan Fast Universal PCR Master Mix (Life Technologies Japan, Tokyo, Japan) on a Thermal Cycler Dice Real Time System II (Takara Bio, Tokyo, Japan). We determined the level of human GHSR mRNA (Hs0269780_s1) using cataloged primers (Applied Biosystems, Foster City, CA). Expression of GHSR was normalized against hypoxanthine phosphoribosyltransferase (HPRT1) mRNA (Hs02800695_m1), and the result is expressed as a relative fold difference.

Cell viability assay
For analysis of cell viability, we used the MTT Cell Viability Assay Kit (Cell Biolabs, San Diego, CA). HLC-1 cells were seeded in 96-well plates at a density of 1 × 10^5 cells/well, and then treated with various concentrations of ghrelin (10^{-2}, 10^{-1}, 1, and 10 μM) and/or PTX (1 μM) for 48 hr at 37°C with 5% CO2. Absorbance data were collected from five wells per condition, and results were normalized as percent of control.

Cell proliferation assay
BrdU assays were performed using the BrdU Cell Proliferation ELISA Kit (Abcam, Cambridge, UK). HLC-1 cells (2 × 10^5 cells/well) were seeded in 96-well plates and cultured with various concentrations of ghrelin (10^{-2}, 10^{-1}, 1, 10 μM) or PTX (1 μM) for 24 h, and then 20 µL of 1 × BrdU solution was added. Absorbance data were collected from five wells per condition, and the results were normalized as percent of control.

TUNEL assay
To evaluate apoptosis of HLC-1 cells, we performed a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using an in situ cell death detection kit, TMR Red (Roche Diagnostics, Basel, Switzerland), and 48 h after cells were exposed to medium containing 1 μM PTX. For quantification of apoptotic cells, one investigator took photos of 13−17 random fields from three cultured dishes per group in a blinded fashion, and then two other investigators independently counted the cells in a blinded fashion in each field. Percentages of TUNEL-positive cells were calculated by dividing the number of TUNEL-positive cells by the number of 4′,6-diamidino-2-phenylindole (DAPI)-positive cells.

Mitochondrial respiration assays
We measured oxygen consumption rate in real time in HLC-1 cells (1.5 × 10^4 cells/well) using an
Influence of ghrelin on lung cancer progression

Discussion

The results of this study provide the first evidence that ghrelin does not affect cancer progression in lung adenocarcinoma. Although a considerable body of in vitro researches indicated that ghrelin might affect cancer progression via several signaling pathways, e.g., PI3K/Akt [9] and Erk1/2 [10], our findings demonstrate that ghrelin treatment did not influence biological activities, viability, proliferation, or resistance to apoptosis in human lung adenocarcinoma HLC-1 cells. In normal cells, ghrelin promotes proliferation by stimulating the PI3K/AKT pathway and MAPK pathways [11], and also increases resistance to apoptosis [12]. The discrepancy in cell proliferation and apoptosis resistance between normal and cancer cells may be in part due to differences in the expression level of the GHSR. In the present study, the GHSR mRNA level was lower in HLC-1 cells than in normal airway BEAS-2B cells. These data suggest that the difference in the level of the GHSR expression may be related to the magnitude of stimulation of proliferation and resistance to apoptosis.

Mitochondrial activity in cancer cells is a pivotal factor in cancer progression [13]. KRAS-mutated lung cancers, which have deficiencies in electron transport and oxidative phosphorylation at the stage of mitochondrial respiration following inactivation of the mitochondrial transcription factor Tfam, exhibit impaired progression [13, 14]. Although ghrelin is thought to activate mitochondrial activity [12], our findings revealed that ghrelin did not affect oxygen consumption rate or individual parameters of mitochondrial respiration, i.e., basal respiration, proton leak, ATP production, maximal respiration, spare respiratory capacity, and non-mitochondrial respiration. These findings suggest that ghrelin has no direct effect on mitochondrial respiration activity in lung adenocarcinoma.

Our findings indicate that ghrelin does not directly increase cancer progression in lung adenocarcinoma cells. Ghrelin represents a promising agent for the safe treatment for cancer cachexia without promoting cancer progression.

Disclosures

The authors declare no conflicts of interest associated with this manuscript.
Fig. 1  Expression of the GHSR in lung epithelial cells and lung adenocarcinoma cells, and the effects of ghrelin on cancer progression

A: Representative profiles of Ghsr-eGFP mouse lung sections double-immunostained for GFP (green) and SP-C (red). GFP+/SP-C+ cells (Ghsr-expressing type II alveolar epithelial cells, arrows) and GFP+/SP-C- cells (Ghsr-expressing type I alveolar epithelial cells, arrowheads) are observed in the alveolar wall. Scale bar: 25 µm.

B: Levels of GHSR mRNA expression (normalized to HPRT1) in BEAS-2B (n = 5) and HLC-1 (n = 5) cells. Data are means ± SE. * p < 0.05.

C and D: Ghrelin (10^-2, 10^-1, 1, or 10 μM) did not significantly increase the viability of cells incubated with or without PTX, or the resistance to PTX-induced apoptosis of HLC-1 cells. Absorbance data were collected from five wells for each cell type and condition, and results were normalized as percent of control. Data are means ± SE. * p < 0.05.

E: Representative profiles of TUNEL staining (red) of PTX-treated HLC-1 cells. DAPI was used to counterstain the nucleus (blue). The numbers of TUNEL-positive cells did not differ significantly in the PTX-treated group. Scale bar: 25 µm.
Influence of ghrelin on lung cancer progression

Fig. 2  The effect of ghrelin on mitochondrial respiration
The oxygen consumption rate of HLC-1 cells pretreated for 12 h with or without ghrelin (10 μM) was measured in real time by a Mito Stress Test in an XFp analyzer. A: Oxygen consumption rates of the HLC-1 cells and (B) individual parameters of basal respiration, proton leak, ATP production, maximal respiration (Max respiration), spare respiratory capacity (spare respiration), and non-mitochondrial respiration (non-mito respiration). Data are means ± SE; n = 3 assay replicates per sample.

References

1. Fearon KC, Glass DJ, Guttridge DC (2012) Cancer cachexia: mediators, signaling, and metabolic pathways. Cell Metab 16: 153-166.
2. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, et al. (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402: 656-660.
3. Akamizu T, Kangawa K (2011) Therapeutic applications of ghrelin to cachexia utilizing its appetite-stimulating effect. Peptides 32: 2295-2300.
4. Chopin LK, Seim I, Walpole CM, Herington AC (2012) The ghrelin axis--does it have an appetite for cancer progression? Endocr Rev 33: 849-891.
5. Lin TC, Hsiao M (2017) Ghrelin and cancer progression. Biochim Biophys Acta 1868: 51-57.
6. Tisdale MJ (2002) Cachexia in cancer patients. Nat Rev Cancer 2: 862-871.
7. Matsubara D, Ishikawa S, Ogumi S, Aburatani H, Fukayama M, et al. (2010) Molecular predictors of sensitivity to the MET inhibitor PHA665752 in lung carcinoma cells. J Thorac Oncol 5: 1317-1324.
8. Rogers GW, Brand MD, Petrosyan S, Ashok D, Elorza AA, et al. (2011) High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. PLoS One 6: e21746.
9. Lien GS, Lin CH, Yang YL, Wu MS, Chen BC (2016) Ghrelin induces colon cancer cell proliferation through the GHS-R, Ras, PI3K, Akt, and mTOR signaling pathways. Eur J Pharmacol 776: 124-131.
10. Tian PY, Fan XM (2012) The proliferative effects of ghrelin on human gastric cancer AGS cells. J Dig Dis 13: 453-458.
11. Mao Y, Wang J, Yu F, Li Z, Li H, et al. (2016) Ghrelin
protects against palmitic acid or lipopolysaccharide-induced hepatocyte apoptosis through inhibition of MAPKs/iNOS and restoration of Akt/eNOS pathways. *Biomed Pharmacother* 84: 305-313.

12. Zhang Q, Huang WD, Lv XY, Yang YM (2011) Ghrelin protects H9c2 cells from hydrogen peroxide-induced apoptosis through NF-kappaB and mitochondria-mediated signaling. *Eur J Pharmacol* 654: 142-149.

13. Zong WX, Rabinowitz JD, White E (2016) Mitochondria and Cancer. *Mol Cell* 61: 667-676.

14. Weinberg F, Hamanaka R, Wheaton WW, Weinberg S, Joseph J, *et al.* (2010) Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity. *Proc Natl Acad Sci U S A* 107: 8788-8793.