Rikkunshito attenuates induction of epithelial-mesenchymal switch via activation of Sirtuin1 in ovarian cancer cells

Ranka Kanda1),* , Yuko Miyagawa1),* , Osamu Wada-Hiraike2), Haruko Hiraike1), Shiho Fukui1), Kazunori Nagasaka1), Eiji Ryo1), Tomoyuki Fujii2), Yutaka Osuga2) and Takuya Ayabe1)  

1) Department of Obstetrics and Gynecology, Teikyo University School of Medicine, Tokyo, Japan  
2) Department of Obstetrics and Gynecology, The University of Tokyo, Tokyo, Japan  

Abstract. Rikkunshito, a traditional Japanese herbal medicine, improves appetite via activation of gastrointestinal hormone ghrelin pathway. The function of ghrelin is mediated by growth hormone secretagogue receptor (GHSR1a), and ghrelin has been known to possess diverse physiological functions including growth suppression of some cancer cells. Considering that increased ghrelin signaling by Rikkunshito could enhance sirtuin1 (SIRT1) activity in nervous system, we aimed to investigate the effect of Rikkunshito in ovarian cancer cells. Ovarian cancer cell lines were treated with Rikkunshito, and cellular viability, gene expressions and epithelial-mesenchymal transition (EMT) status were investigated. To investigate the involvement of SIRT1 by Rikkunshito in SKOV3 cancer cells, endogenous expression of SIRT1 was depleted using small interfering RNA (siRNA). Treatment with Rikkunshito elevated ghrelin, GHSR1a and SIRT1, while cellular viability was decreased. The treatment of Rikkunshito also inhibited cellular migration and invasion status in a dose-dependent manner, and these effects were translated to the enhanced EMT status, although the role of SIRT1 was not determined. Our study revealed a novel function of Rikkunshito in enhancing EMT status of ovarian cancer cells. Therefore, we would like to propose that Rikkunshito may be used as a novel adjunctive therapy in chemotherapy of ovarian cancer because platinum-based chemotherapy frequently used for the treatment of ovarian cancer inevitably impairs appetite.  

Key words: Traditional Japanese herbal medicine, Rikkunshito, Ghrelin, Sirtuin1, Epithelial-mesenchymal transition

Rikkunshito (RKT), a traditional Japanese herbal medicine called Kampo, has been used for the patients suffering from anorexia. RKT improves appetite via activation of gastrointestinal hormone ghrelin [1, 2]. Beside this, previous report has shown that RKT significantly improve chemotherapy-induced nausea, vomiting, and anorexia in gynecologic malignancy cancer patients [3]. And then, opportunity for the use of RKT has been increasing. RKT enhances the secretion of ghrelin, a 28-amino acid stomach-derived peptide, is predominantly produced by the stomach.  

Ghrelin is the endogenous ligand for growth hormone secretagogue receptor (GHSR). It has been reported that GHSR1b, isoform of GHSR, generated by alternative splicing has no bioactivity because of inability to bind any growth hormone secretagogue [4]. Namely, only GHSR1a is involved in the signaling pathway and activities of ghrelin [5]. Both ghrelin and GHSR1a have been detected in various tissue, including the lung, testis, ovary, breast, endometrium [6]. Ghrelin mainly stimulates growth hormone (GH) secretion from the anterior pituitary glands and upregulates appetite. Besides its central function, ghrelin is also involved in improving cardio-vascular events, anti-inflammatory effects, modulating cell proliferation, glucose and lipid metabolism [7].  

According to the previous report, the increasing signaling pathway of ghrelin by RKT prolong the life time via activation of sirtuin1 (SIRT1) [8]. SIRT1 is one of the sirtuin family, known as a longevity gene and is activated by calorie restriction. It functions as a deacetylase, interacts with transcription factors such as p53 and nuclear factor kappa B. Its biological functions are various, apoptosis, stress resistant, glycolipid metabolism, increase of insulin secretion, formation of memory and circadian rhythm [9].
Recently, it has been reported that ghrelin suppresses ovarian cancer cells via induction of apoptosis and autophagy [10, 11]. Moreover, it remains possible that SIRT1 suppress the progression of some cancer [12, 13]. Therefore, we investigate the effect of RKT, enhances the secretion of ghrelin, and the association between ghrelin and SIRT1 in ovarian cancer cells.

Materials and Methods

Cell lines and Cell culture
An ovarian cancer cell line SKOV3 (HTB-77, ATCC, Manassas, USA) that express GHSR was used in our study. SKOV3 cells were cultured in McCoy’s 5A Medium (Gibco, NY, USA) containing 10% FBS, 1% Zell Shield.

Chemical and antibody
According to the previous reports [8, 14], Rikkunshito (Tsumura, Tokyo, Japan) were dissolved in DMSO (dimethyl sulfoxide) and appropriate concentration of RKT (0, 50, 100 μg/mL) was applied. Both Anti-β-actin (sc-47778, mouse monoclonal antibody) and anti-vimentin (sc-6260, mouse monoclonal antibody) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-SIRT1 (ab110304, mouse monoclonal antibody) was purchased from abcam (Cambridge, Cambridgeshire, UK). Both E-cadherin (610181, mouse monoclonal antibody) and anti-N-cadherin (610920, mouse monoclonal antibody) were purchased from BD (Franklin Lakes, NJ, USA).

Cell viability assay
We performed cell viability assay using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan). SKOV3 were seeded in 96-well plate at a density of 1 × 10^3 cells/well in 96-well plate in 100 μL of medium with 10% serum after the addition of Rikkunshito (0, 50 μg/mL, 100 μg/mL). Subsequently, we added 10 μL of reagent to each well. After incubation for 3 hours, the absorption to each sample were measured at 570 nm using a microplate reader. We repeated three times independently and analyzed the results.

Wound healing assay
We performed wound healing assay using CytoSelect TM 24-Well Wound Healing Assay (CELL BIO LABS, San Diego, CA, USA) following the instructions in the manual. Invasion plate were added 0.5 mL serum-free medium in both insert and well and incubated for 2 hours to rehydrate only invasion chamber. Control chamber was not needed to rehydrate. After incubation, we remove the medium. Cells (0.25 × 10^6) were seeded with 500 μL medium including serum on insert and we added medium including serum on well. Samples were incubated for 48 hours, and we remove medium using a cotton swab several times. Samples were stained by Diff-Quick solution. We counted cells in several fields by micro-
Small interfering RNA (siRNA) forward transfection

One day before transfection, cells seeded in 24-well plates without antibiotics. Cells were added the RNAi duplex-Lipofectamine™RNAiMAX complexes to each well and then incubated the cells 24 hours at 37°C in a CO₂.

Statics analysis

Data are presented as the mean ± standard error (SE) from at least three independent experiments. Statistical significance was determined using Student’s t-test to compare two groups or one-way ANOVA with Turkey’s post hoc test to compare four groups, using the GraphPad Prism 6 software (GraphPad, San Diego, CA). A p values less than 0.05 were considered statistically significant.

Results

Rikkunshito inhibits cell viability of ovarian cancer cells and upregulated the expression levels of both GHSR1a and ghrelin in a dose-dependent manner

We first confirmed the GHSR1a expression status in SKOV3 (Fig. 1A) and then investigated the effect of RKT by performing MTT assay. We incubated SKOV3 cells with RKT of different concentration. We empirically and preliminary determined the dose of RKT considering the previous report [8, 18], and RKT slightly but significantly decreased the cell number in a dose-dependent manner (Fig. 1B). Next, we confirmed whether RKT could influence on both ghrelin and its receptor, and RKT increased the mRNA expression level of ghrelin and its receptor with a dose-dependent manner (Fig. 1C and 1D). Moreover, we confirmed the results using ID8 cells established from C57/BL6 mouse ovarian surface epithelial cancer cell line (data not shown).
Rikkunshito inhibits the migration and invasion ability of SKOV3

Next, we aimed to investigate antitumorigenic properties of RKT using wound healing assay to assess migration ability of RKT, and treatment of RKT for 24 hours downregulated the migration ability of SKOV3 cells (Fig. 2A). Thus, we hypothesized that RKT could inhibit migration and invasion capacity. In concordance with this hypothesis, RKT suppressed migration and invasion ability in a dose-dependent manner (Fig. 2B and 2C).

Rikkunshito induces SIRT1 activity and suppresses N-cadherin switch

We then examined the mechanism of these antitumorigenic functions of RKT, and a longevity gene SIRT1 was supposed to be a candidate [8]. In addition to SIRT1 as a possible driver gene of RKT function, epithelial-mesenchymal transitional markers such as E-cadherin, N-cadherin, and vimentin were investigated. As expected, RKT treatment increased the protein level of SIRT1 (Fig. 3A). We further showed that the protein level of E-cadherin increased dose-dependently (Fig. 3A). In contrast, RKT treatment at 100 μg/mL decreased the protein levels of N-cadherin (Fig. 3B) and vimentin (Fig. 3C).

Knockdown of SIRT1 in SKOV3 decreased ghrelin expression to induce EMT and enhance invasion ability

We further investigated the role of SIRT1 in SKOV3 ovarian cancer cells. To test this, siRNA-mediated knockdown of SIRT1 was performed. We confirmed that the expression of SIRT1 was downregulated by depleting SIRT1 (Fig. 4A). We further investigated directly association between SIRT1 and ghrelin. Knockdown of SIRT1 with RKT treatment tended to decrease the expression of ghrelin (Fig. 4B). Knockdown of SIRT1 significantly decreased ghrelin expression compared to negative control under condition of RKT treatment, although it was not significantly difference among RKT treatment.

The effect of SIRT1 on ovarian cancer cell viability
was analyzed by MTT assay at 48 hours after SIRT1 knockdown. SIRT1 knockdown upregulated cell viability compared to negative control (Fig. 4C). And we further investigated whether SIRT1 influence invasion ability in SKOV3 cells and we showed that knockdown of SIRT1 augmented invasion ability (Fig. 4D).

Moreover, knockdown of SIRT1 trended to upregulate the protein level of vimentin and N-cadherin compared to negative control (Fig. 4E), although there was no significant difference between the knockdown of SIRT1 and negative control.

**Discussion**

To elucidate effect of RKT, we focused on gastrointestinal hormone ghrelin. Ghrelin is generally known to mediate its actions via specific receptor, the GHSR1a. It has been reported that GHSR1a is expressed in normal ovary, fallopian tube, and serous in benign and malignancy tumors [19]. First, we confirmed the expression of GHSR1a and ghrelin using ovarian serous carcinoma cell lines such as SKOV3, MES-OV and mouse ID8 cells, but due to the limitation of assays, SKOV3 cell line was selected for further assays. Moreover, a previous report also indicated that EMT can be well observed using SKOV3 cells [20].

We showed that RKT dose-dependently increased the expression of GHSR1a and ghrelin signaling and contributed to attenuate the ovarian cancer cell growth. Moreover, RKT dose-dependently attenuated migration and invasion in vitro level. The majority of reports on ghrelin invasion ability in some cancer cells is that ghrelin promotes invasive and migration ability [21, 22]. It is important to note that we performed wound healing, invasion and migration assay to evaluate the effect of RKT, not directly that of ghrelin.

Since SIRT1 knockdown increased invasive ability (we describe further details later), we consider that SIRT1 mainly reduced invasion and migration ability.

The variety of ghrelin function has gained popularity...
Ghrelin is involved in invasion, migration, apoptosis, and proliferation in cancer. Some reports indicate ghrelin suppressively influence for cancer, while others indicate the opposite mechanism [23]. At present, the mechanism of ghrelin for cancer are still unknown.

We further elucidated cancer suppression mechanism of RKT, we focused on EMT (Epithelial to mesenchymal transition). The pathological activation of EMT in cancer cells is thought to be particularly involved in the process of cancer invasion and metastasis. Various external stimuli cause the transition from epithelial cancer cells to mesenchymal cells, that is, EMT. Generally, expression of decreased epithelial cell markers (E-cadherin) and increased mesenchymal cell markers (vimentin, N-cadherin, fibronectin) is widely used as an indicator of induction of EMT in cancer cells. Therefore, cancer cells might enhance invasion and metastasis by induction of EMT. Our research revealed that RKT suppressed induction of EMT and enhanced SIRT1 activity. Morphological changes of cells after addition of RKT was confirmed to some extent but it was difficult to draw conclusive data (data not shown).

We also found that SIRT1 is involved in suppression of cancer growth by knockdown SIRT1. SIRT1 plays a role as a NAD⁺-dependent deacetylase and control a wide range of physiological functions by interacting with various proteins in vivo. SIRT1 is paid attention as a target for cancer treatment. SIRT1 might increase in cancer cells and reduce activation of p53 against DNA damage to promote anti-cancer drug resistance and human tumorigenesis [24-26]. On the other hand, SIRT1 suppress cancer cells by activating gene repair system and SIRT1 overexpression attenuate cancer development [12, 13]. It is presumed that SIRT1 has two aspects, suppressor gene and oncogene. One is inhibitory action on cancer cells, the other is promotion action on cancer cells and resistant to chemotherapy. It is considered that SIRT1 has different effect in various organs, tissues, and cells depending on the expression level of SIRT1.

Fig. 4 Role of SIRT1 in enhancement of EMT in SKOV3 cells.

(A) The expression level of SIRT1 was downregulated by knockdown of SIRT1 by siRNA.
(B) The association between SIRT1 and ghrelin under the treatment of RKT were investigated by knockdown of SIRT1.
(C) The cellular viability after depletion of endogenous SIRT1 was enhanced compared to negative control.
(D) Knockdown of SIRT1 tended to enhance the rate of SKOV3 mobility, although it was statistically insignificant.
(E) The expression levels of EMT markers were investigated after knockdown of SIRT1 by siRNA.

(*p < 0.05)
NC: negative control

worldwide in recent years. Ghrelin is involved in invasion, migration, apoptosis, and proliferation in cancer.
The present study revealed that RKT attenuate induction of EMT via activation SIRT1 to suppress cancer growth. We intendedly depleted SIRT1 expression to investigate two associations; the first is association between ghrelin and SIRT1 under the condition of RKT treatment, the second is association between SIRT1 and EMT. SIRT1 knockdown tended to decrease ghrelin expression, although it was not significantly difference under condition of RKT treatment. In other words, we suggested that ghrelin itself may activate SIRT1. This result is consistent with a previous report [8].

Moreover, we confirmed that SIRT1 knockdown did not significantly upregulated vimentin and N-cadherin, representative EMT markers. Namely, SIRT1 was not directly involved in EMT functions and can be interrupted as other factors might be involved.

Concerning the relationship between SIRT1 and EMT, SIRT1 suppress EMT in cancer metastasis [27]. However, SIRT1 induces EMT by cooperating with EMT transcription factors and enhances cancer cell migration and metastasis [28]. The results of obtained so far are controversial.

In recent years, there have been many reports on the anti-tumor effect of Kampo medicine. Some reports also suggested that Juzentaihoto (JTT) inhibited the growth of cancer as well as RKT [29]. The component of JTT might contribute to suppress cancer via activation of an immune system [30]. From the previous reports, it is presumed that RKT might have biological activity similar to that of JTT.

Recent investigations have demonstrated that RKT is effective for the patients with chemotherapy-induced anorexia [3]. However, what we are concerned here is that RKT slowly shows the effective action, is not anticancer drug which blocks cell growth and may kill cancer cells. Therefore, it is expected to be adjuvant applied clinically for patients with ovarian cancer in future.

In summary, the present study demonstrated that RKT attenuates induction of EMT via action of SIRT1 in ovarian cancer cells. We would like to research not only pleiotropic function of RKT in vivo level, but also the possibility the use of RKT with adjuvant to chemotherapy in the future.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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