The Extracts from *Allium hookeri* induces p53-independent Apoptosis through Mitochondrial Intrinsic Pathways in AGS Human Gastric Carcinoma Cells

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**Abstract**

*Allium hookeri* is a traditional plant to treat inflammatory diseases in India and Myanmar. *Allium hookeri* has anti-cancer and anti-microbial properties. However, till date, apoptotic mechanisms of *Allium hookeri* extract (AHE) were not investigated yet. In the present study, we aimed to investigate the apoptotic effects of AHE in AGS human gastric carcinoma cells. We confirmed the anti-proliferative activity and apoptotic effects of AHE by MTT assay and Annexin-V staining. In addition, treatment with AHE reduced the expression level of p-Akt. Akt plays an important role in cancer cell survival, growth, and division. Down-regulates apoptosis-mediated proteins, such as anti-apoptotic proteins. Moreover, AHE increases expression level of tumor suppressor p53 and pro-apoptotic proteins. We treated LY294002 (inhibitor of Akt) and rapamycin (inhibitor of mTOR), pifitrin-α (p53 inhibitor) to determine the relationship between signal transduction of proteins associated with apoptosis. Taken together, our results indicate that AHE could induce apoptosis in AGS human gastric carcinoma cells.

**Keywords:** *Allium hookeri*; Apoptosis; Gastric carcinoma cells; Tumor suppressor; Anticancer chemotherapy

**Introduction**

Cancer is one of the biggest causes of death worldwide, and the death rate from cancer continues to increase in the developed countries and other countries [1]. Today, every third person suffers from cancer [2]. Among various cancer types, stomach cancer is known to be the most frequent cancer regardless of gender. Recent advances in cancer diagnosis and treatment have emphasized the importance of early screening to increase the survival rate of stomach cancer patients [3]. If cancer is diagnosed early, it will be treated with gastric mucosectomy or surgery using a laparoscope. However, in the case of metastasis to other parts of the body, the patient will be treated with anticancer chemotherapy without becoming an indication for the operation. As a result, stomach cancer patients may experience post-removal dumping syndrome, nutritional deficiency, and anemia during the treatment process, and anticancer chemotherapy may cause misdiagnosis, vomiting, constipation, diarrhea, hypostosis, and bone marrow functions.

In previous research, it has been pointed out that this causes patients with stomach cancer to experience physical and psychological pain, leading thus to reduced quality of life [4]. For that reason, many studies have recently been conducted to develop new physiologically active substances based on the efficacy of various natural substances that are harmless to humans, as the natural extracts can inhibit and regulate the signaling molecules associated with the growth and proliferation of cancer cells [5]. Among such natural products, *Allium hookeri*, which has a variety of physiological activities, such as anti-inflammatory property, is a vegetable mainly consumed in the Himalayas where it is used for various inflammation-related and cancer diseases. Now that *Allium hookeri* is brought Korea, there is a growing interest in its ingredients and efficacy [6,7]. It was reported that the anti-inflammatory effect of *Allium hookeri* prevents cell growth and mutagenesis and induces apoptosis (death of a cell) in cancer cells [8]. Therefore, seeking for a new anticancer substance using such natural materials, research is currently underway on which natural extracts induce apoptosis and thus have anticancer effects [9]. Apoptosis is a very important process, because it shrinks cells, condenses nuclei, and forms cell membrane bubbles due to the programmed cell death, and if the cells do not die properly, mutations of some cells will be promoted with cancer [10].

Apoptosis’s pathways are divided into extrinsic pathways and intrinsic pathways especially in mitochondria, which play an important role in the intrinsic pathways of apoptosis. The method is influenced by the relative ratios of Bcl-2, an anti-apoptotic protein, and Bak and Bax proteins of pro-apoptotic protein [11]. Akt (serine/threonine kinase), one of the regulating proteins of apoptosis intrinsic pathways, regulates cell proliferation, differentiation, and growth, and inhibits Bak and Bax [12]. The mTOR activated by Akt is involved in tumor formation and is regulated in cell growth and proliferation [13,14]. In addition, mTOR induces ubiquitation of p53 by phosphorylating MDM2 (Murine Double Minute 2) and affects cell growth [15]. The affected p53 adjusts gene expression involved in cell death with a transcription factor [16]. Therefore, caspase cascade is induced under the influence of the apoptosis intrinsic pathway, and apoptosis is induced through the activity of caspase-3 [17]. Therefore, in the present study, we sought to determine the expression of the anticancer activity mechanism in the AGS human gastric cancer cells, and whether or not the suppression of the proliferation of the protein would be caused by intrinsic apoptosis.

**Materials and Methods**

**Reagents**

*Allium hookeri* (AHE) were purchased from Heungdang company

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Received July 11, 2018; Accepted August 06, 2018; Published August 08, 2018


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The AHE was refluxed with 95% ethanol for 24 hr. The extract was concentrated to remove the ethanol. The extract was dissolved in DMSO prior to treatment and refrigerated at -18°C. The Muse TM Annexin V apoptosis detection kit was obtained from Merck (Darmstadt, Germany). Specific antibodies that recognized mTOR, Akt, p53, Bax, Bak, procaspase-3, Bcl-2, and β-actin are obtained from Cell Signaling Technology (Beverly, USA). LY294002 (Akt inhibitor) and Rapamycin (mTOR inhibitor), Pifithrin-α (p53 inhibitor) was purchased from SIGMA (Spruce Street, St Louis, USA).

Cell culture
AGS Human Gastric Carcinoma Cells were obtained from the American Type Culture Collection (ATCC, USA). The cells were grown in RPMI-1640 medium (Hyclone, USA) containing 10% fetal bovine serum (Hyclone, USA) and 1% antibiotics (100 mg/L streptomycin with 100 U/mL penicillin) in a 5% CO2 atmosphere at 37°C. The cells were suspended by Trypsin-EDTA (Hyclone, USA) and separated at 1 × 10^6 cells/mL per plate, every 48-72 hours.

MTT assay
Cells seeded on 96-well micro plates at 4000 cells/well were incubated with the test compounds for indicated time period. Respectively medium was removed and then incubated with 40 μl of MTT solution (2 mg/mL MTT in PBS) for 1 h. MTT is converted to a blue formazan. Absorbance was determined using an auto reader.

Determination of apoptosis by Annexin V staining
The cells were seeded at 1 × 10^6 cells/mL in plate and incubated for 24 h. The cells were treated with the AHE for 24 h in a 5% CO2 atmosphere at 37°C. The inhibitor was pre-treated for 30-120 min before treating AHE. Total cells were harvested, collected by centrifugation, washed with PBS. Cells were stained with Annexin V for 20 min. apoptosis was analyzed using a Muse TM Cell analyzer (Merck, Germany).

Western blotting
The cells were seeded at 1 × 10^6 cells/mL plate. Then, the cells were treated with the AHE for 24 h in a 5% CO2 atmosphere at 37°C. The inhibitor was pre-treated for 30-120 min before treating AHE. The cells were scraped with RIPA lysis buffer. The primary antibody was then added following by overnight at 4°C, following the addition of the secondary antibody was added following by 30-120 min.

Statistical analysis
MTT assay was statistically analyzed using t-test (SPSS Inc.; Chicago, USA). A value of P<0.05 was indicated to a statistically significant difference.

Results
Impact of AHE on AGS human gastric cancer cell proliferation
MTT assay after processing AHE (15, 35, 35, 70, 105, 140, and 170 μg/mL) was applied to AGS human gastric cancer cells to confirm the effect of AHE on AGS and gastric cancer cell growth rate. The results show that, when the AHE was treated for 24 hours, the cell proliferation was suppressed in a dose-dependent manner (Figure 1A). The results of measuring the cytotoxicity of AHE to fibroblast cells of normal cells confirm that the proliferation rate of fibroblast cells was maintained at least 90% (Figure 1B). When apoptosis was induced, the cells were broken up, the cells were condensed in chromatin, the bubbles formed in the plasma membrane, and the cells were divided into cells. Lamellipodia and filopodia, which play important roles in cell migration, were also lost and the cells lost adhesion. Therefore, we observed that such morphological changes in apoptosis and cell migration were not observed in fibroblasts.

Figure 1: Cytotoxic and anti-proliferation Effects of AHE in AGS human gastric carcinoma cells (A) Cells were treated with variable concentrations of AHE (15~170 μg/ ml) for 24 hr. Cell viability was measured by MTT assay. The statistical analysis of the data was carried out by use of a t-test. * means p<0.05, ** means p<0.01 and *** means p<0.001 was compared to control. N.S. means not significant (each experiment, n=3). (B) MTT assay evaluated cell viability. The statistical analysis of the data was carried out by use of a t-test. N.S. means not significant (each experiment, n=3). (C) Phase Contrast Image-Based Monitoring of Apoptosis Induction (X400 magnification, Axiovert 100, Zeiss, Germany).
proliferation were inhibited when AHE was treated with AGS human gastric cancer cells. As a result, as shown in Figure 1C, we were able to see that the cells in the control group, which did not process anything, were relatively dense, uniform, and normally attached. It was confirmed that the higher the concentration of AHE, the lower the density of the cells, and the more cells become irregular [18].

**Determination of the Application of AGS human gastric cancer cells by AHE**

Early apoptosis occurs in the translocation of phosphatidylserine, an early sign of programmed cell death and the cell membrane remains undamaged in the process of translocation [19,20]. To distinguish whether the death of AGS stomach cancer cells under AHE processing is caused by apoptosis or necrosis, we measured the level of intracellular phosphatidylserine (PS) expression, which is considered to be the early stage of apoptosis development using Annexin V (Figure 2). As shown in Figure 1R, the ratio of Annexin V-positive cells was low in the control (5.9%). However, as the concentration of AHE increased, the percent of annexin V-positive cells increased as well: increasing the concentration of 23.17% (70 μg/mL), 26.20% (105 μg/mL), 31.90% (140 μg/mL). Throughout this process, we observed that the treatment of AHE and the inhibition of the growth of AGS human gastric cancer cells were caused by apoptosis.

**Confirmation of apoptosis intrinsic pathway-related protein expression by AHE**

Mitochondria play an important role in apoptosis. Intrinsic pathway and Bcl-2 family of proteins also play an important role in maintaining its membrane potential [21]. Bcl-2 and Bcl-XL, which are anti-apoptotic proteins, are moved to maintain the stability of the mitochondria membrane and the pro-apoptotic proteins Bax and Bak move in the mitochondrial membrane to play a role of increasing permeability. The caspase cade-inducing caspase-3 is activated to decompose the PARP protein. Therefore, in the present study, Western blotting was performed in order to confirm the degree of expression of not only Akt, mTOR, and p53 proteins, which are the upstream regulators of apoptosis intrinsic pathway, but also affected downstream regulators. As shown in Figure 3, the reduction of p-Akt, p-mTOR and Bcl-2, Bcl-XL and pro caspase-3, which are anti-apoptotic proteins involved in cell proliferation, was confirmed. An increase in Bax, Bak, and cleaved-PARP of p53 and pro-apoptotic protein of the cancer suppressor gene was also confirmed. PARP, a protein decomposed by caspase-3, maintains the stability of DNA repair and genes. If a fragment is cut, it will lose its function. Therefore, it is possible to know that apoptosis by caspase activity is induced by the expression of the cleared-PARP [22,23]. In conclusion, we could learn that AHE induces apoptosis through an intrinsic pathway in AGS human gastric cancer cells.

**The effects of p53 and Akt and mTOR inhibitors on AGS human gastric cancer cells growth**

In order to confirm the relationship between AHE-induced cytotoxicity and apoptotic pathways, after the treatment of LY294002 (Akt inhibitor) or rapamycin (mTOR inhibitor), and the co-treatment of pifitrin-α (p53 inhibitor) by AHE were performed. We found that the AHE treatment group, as well as the LY294002 and the rapamycin treatment group showed a similar decrease as compared to the control. Moreover, the pifitrin-α treatment group did not show a significant difference with the control group. However, if pifitrin-α and AHE were co-treated, Despite the treatment of Pifitrin-α, the number decreased in the same way as in the AHE treatment group (Figure 4). These results mean that AHE induces cytotoxicity and apoptosis through Akt, mTOR dephosphorylation, and p53 independent pathway downregulation.

**Confirmation of apoptosis effect and apoptosis intrinsic pathway-related protein expression by p53 and Akt and mTOR inhibitor**

In order to investigate the apoptosis effect of AGS human gastric cancer cells through intrinsic pathway, Annexin-V staining analysis was conducted using flow cytometry (Figure 5). Furthermore, as shown in Figure 6, we confirmed the control relation between the upstream regulators (p-Akt, p-mTOR and p53) and downstream regulators...

Figure 3: AHE regulates the expression of apoptotic and anti-apoptotic proteins. The expression of mTOR, cleaved PARP, Akt, p53, pro-caspase 3, Bcl-2, Bak, Bax and β-actin were analyzed by Western blot analysis.

Figure 4: Effects on cell proliferation and the control of the signal proteins via inhibition of Akt, mTOR, p53. Cells were treated with 20 μM LY294002 (Akt inhibitor) and 100nM Rapamycin (mTOR inhibitor), 20 μM Pifitrin-α (p53 inhibitor), AHE for 24h. (A) Cells viability was measured by MTT assay. The statistical analysis of the data was carried out by use of a t-test. * means p<0.05, ** means p<0.01 and *** means p<0.001 compared to control. N.S. means not significant (each experiment, n=3).

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Figure 5: Apoptotic effects of different concentration AHE and signal protein inhibitor were evaluated by the Muse™ Annexin V and Dead Cell assay. (A) Apoptotic effects Cells treated LY294002 (Akt inhibitor), Rapamycin (mTOR inhibitor), pifitrin-α (p53 inhibitor) and AHE were evaluated by the Muse™ Annexin V and Dead Cell assay. Data analyzed by flow cytometry (B) Annexin V staining was showed by five categories: live, necrosis, early apoptosis, late apoptosis and total apoptosis.

Figure 6: Protein level was indicated treated LY294002, Rapamycin, pifitrin-α and AHE. The expression of mTOR, cleaved PARP, AKT, p53, pro-caspase 3, Bcl-2, Bak, Bax and β-actin were analyzed by Western blot analysis.
Discussion and Conclusion

Stomach cancer is the most common cancer worldwide, including Korea [25]. Therefore, treatment methods that induce apoptosis are urgently needed to treat stomach cancer. In the present study, we investigated the apoptosis effect of extract from *Allium hookeri*, a plant species that proved to be effective in cancer treatment in the previous literature [26]. Akt signaling pathway is frequently activated in cancers including glioblastoma multiforme (GBM), Ras signaling pathway where this kinase network regulates survival [27,28]. We showed that AHE induces apoptosis by inhibiting Akt signaling pathway. Akt(serine/threonine kinase) mediates of PI3K-initiated signaling pathway by phosphorylating and inhibiting death-inducing proteins and blocks phosphorylates mTOR, MDM2 [29,30]. Phosphorylated-mTOR has emerged as a major regulator of cell proliferation and protein synthesis [31,32]. Previous studies have shown that natural substances inhibit Akt, mTOR induce apoptosis through the intrinsic pathway [33,34]. Furthermore, Phosphorylated-MDM2 inhibits the transcriptional activity of p53 and more importantly, promotes its apoptosis by intrinsic pathway [30,35,36] we confirmed that AHE can induce apoptosis through its downregulation of Akt and mTOR whereby proteins associated with intrinsic pathway work by p53-independent pathway [37]. First, we demonstrated the cytotoxicity effect of AHE through the MTT assay. As a result, AHE significantly reduced viability of cells in the dose-dependent manner. In addition, we detected apoptotic cells using Annexin V staining and found that treatment of AHE on AGS human gastric cancer cells changes the number of apoptotic cells in the dose-dependent manner. Furthermore, in order to investigate if apoptosis goes through the intrinsic pathway, we used Western blotting to confirm the degree of expression of not only Akt, mTOR, and p53 proteins, which are the upstream regulators of apoptosis, Intrinsic pathway, but also affected downstream regulators [38,39]. Then, we were able to observe that the reduction of p-Akt, p-mTOR and Bcl-2, Bcl-XL and pro caspase-3, which are anti-apoptotic proteins involved in cell proliferation. An increase in Bax, Bak, and cleaved-PARP of p53 and pro-apoptotic protein of the cancer suppressor gene involved in cell proliferation. An increase in Bax, Bak, and cleaved-PARP of p53 and pro-apoptotic protein of the cancer suppressor gene were also confirmed. Additionally, to confirm the effect of Akt and mTOR and p53 in AHE-induced apoptosis, we induced Akt, mTOR and p53 inhibitors. The LY294002 treated group, rapamycin-treated group, and AHE single-treated group were induced in similar number of cell toxicity and apoptosis. Even when co-treated with AHE and pifithrin-α, the groups had reduced apoptosis (pifithrin-α blocks the group, and AHE single-treated group were induced in similar number of mTOR and p53 in AHE-induced apoptosis, we induced Akt, mTOR and p53 inhibitors. The LY294002 treated group, rapamycin-treated group, and AHE single-treated group were induced in similar number of cell toxicity and apoptosis. Even when co-treated with AHE and pifithrin-α, the groups had reduced apoptosis (pifithrin-α blocks the expression of p53 and reduces apoptosis [40]). It was not inhibited and apoptosis was normally induced. These results mean that AHE induces cytotoxicity and apoptosis through Akt, mTOR dephosphorylation, and p53-independent pathway downregulation.

Acknowledgements

The study was supported by the 2018 Forbio Ltd. Research Fund.

References


