Research Article

Anticancer Activity Induced by a Rare Ginsenoside Compound K in Human Neuroblastoma

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Abstract | Neuroblastoma is the most common tumor in children and is still a carcinoma, which requires discovery of new therapeutic agents as were in cases of other cancers. Although the anticancer activity of a rare ginsenoside compound K (CK) against various cancers has been reported, there are few studies on neuroblastoma. In this study, cell viability and cell migration assay were tested and the expression of 17 genes in related signaling pathways was analyzed to determine the potential of CK as an anticancer agent for neuroblastoma. For the study of human neuroblastoma, SK-N-MC cells were studied. IC50 values were 33.06 μM and at 40 μM and 60 μM of CK, the wound coverage rates were 11.5% and 0.5%, indicating the cell migration was strongly inhibited. Among the apoptosis-related genes of BAK1, caspase -3, -8, -9, and -12, the expression of caspase 8 was decreased but the expression of caspase 9 and caspase 3 increased, suggesting that the intrinsic signaling pathway was highly activated. Throughout the analyses of cell cycle-related genes, CD1, CDK4, mTOR, Raptor, H-, K-, and N-Ras, the expression of CD1 was decreased, but the expression of other genes was slightly increased. This suggests that CK may act as an inhibition of CD1/CDK4 expression for G1 arrest. Moreover, the expression of p53, p21, MMP2, and MMP9 related to cancer metastasis and invasion was significantly increased or decreased in the CK-treated group, showing that CK properly and antagonistically works on anticancer activity. The expression of angiogenesis-related gene of VEGF unexpectedly was increased. In conclusion, this study confirmed that ginsenoside CK is a new anticancer candidate with excellent anticancer activity inducing cell cycle arrest, apoptosis and in some extent cell migration/metastasis inhibition for neuroblastoma.

Keywords | Neuroblastoma, Compound K , Anticancer

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INTRODUCTION

Ginseng (Panax ginseng) is a perennial plant belonging to the Asteraceae family, named Panax ginseng CA Meyer by CA Mayer in 1843, meaning "cures for all". It contains various nutrients and physiologically active components such as ginseng saponin, phenol, alkaloid, essential oil, free sugar, amino acid, organic acid, carbohydrate, and vitamin etc (Park, 2019). Among them, ginsenoside (GS), called ginseng saponin, is recognized as the most important pharmacologically active ingredient of ginseng through animal experiments and clinical studies and has

been used for medicinal purposes for a long time especially in oriental medicines (Lee et al., 2019).

The saponin of ginseng is called ginsenoside to distinguish it from saponin of other plants, and this name comes from glycosides isolated from ginseng (Park, 2019). About 150 types of ginsenosides have been identified in roots, fruits, leaves, and sprouts and processed ginseng products (Oh and Kim, 2016). GS is named ginsenoside Rx according to the type, "R" means Radix or Root, and X is named in alphabetical order of a, b, c from bottom to top according to the Rf value appearing on TLC (Sjibata, 1966). GS is largely classified into protopanaxadiol (PPD)-type GS and protopanaxatriol (PPT)-type GS according to the number of hydroxyl groups (-OH) attached to the aglycone group (Park, 1996).

The PPD-type GS includes Compound K (CK), F2, Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd and Rh2, and PPD-type GS includes F1, Rg1, Rg2, Rf, Re, and Rh1 (Kim et al., 2018). GS is sometimes divided into major GS and minor GS depending on the amount contained in ginseng. Natural ginseng contains more than 90% of major GS such as Rb1, Rb2, Rc, Re, Rf (Christensen, 2008), and Rg1, but minor GS such as Rg3, Rh1, Rh2, F1, F2, and CK contain trace amounts (Kim, 2018). Among them, F2 and CK are not detected in natural ginseng, but are found in organs or blood through gastrointestinal metabolism after oral ingestion of PPD-type GS (Santangelo, 2019; Yang, 2015).

Major GS is not absorbed into the body after ingestion because it is linked to a polymer component, but it is easily absorbed when it is hydrolyzed and converted into minor GS such as Rg3, Rh1, Rh2, and CK by the unique microorganisms living in the human intestine. As for the GS conversion method, conversion by acid, base, heat, microorganisms, and enzyme, etc. have been reported. Recently, conversion method using microorganisms or enzymes has been actively performed (Lim, 2011). In addition, it was found that these minor GS exhibit excellent medicinal effects compared to major GS in anticancer activity, immune increase, blood improvement, and dementia prevention (Lim, 2011).

Neuroblastoma is a malignant tumor derived from primordial neural crest cells and is one of the most common tumors occurring in childhood. It is the second most common cancer after leukemia, central nervous system tumor, and lymphoma, and accounts for about 10% of all childhood cancers, with an incidence of 1 in 100,000 people every year (Gang et al., 2008). Although recent improvements in neuroblastoma treatment have increased the 5-year survival rate for low-risk disease to more than 90%, the high-risk group, which is more than 40% of patients, has a 5-year survival rate of less than 50% despite the introduction of intensive care therapy. In addition, the prognosis of recurrent neuroblastoma is less than 10% with a 5-year survival rate, and there is an urgent need for new therapies for the treatment of this disease (Swift, 2019).

Ginsenoside CK has been found to have anti-inflammatory, anti-allergic, anti-diabetic, neuroprotective, anti-aging and hepatoprotective effects (Yang et al., 2015) as well as apoptosis, cell proliferation, and angiogenesis inhibitory effects on various cancer cells such as liver cancer (Zhang et al., 2020), lung cancer (Chen et al., 2019), colon cancer (Yao et al., 2018), prostate cancer (Lee et al., 2021), and breast cancer (Ahn, 2018), but the anticancer effect on neuroblastoma cells has not yet been reported.

Therefore, this study was conducted to investigate the anticancer activity by treating the neuroblastoma SK-N-MC cells with compound K and analyzing the cell proliferation, migration, and apoptotic pathway.

MATERIALS AND METHODS

A rare ginsenoside Compound K

The molecular weight and chemical formula of CK are 622.86 g/mol and C36H62O8, respectively (Oh and Kim 2019). Ginsenoside CK powder was dissolved in DMSO to make a 100 mM stock solution, diluted with DMSO according to experimental conditions. The CK solution was mixed at a ratio of 1 µL per 1 mL of culture medium immediately before cell treatment.

Cell Culture

SK-N-MC cell lines (KCLB 30010) were obtained from the Korean cell line bank. The cells were cultured in high glucose DMEM (Biowest) supplemented with 10% (v/v) fetal bovine serum (FBS; Biowest) and 1% antibiotics, Penicillin/streptomycin (100 unit/mL, Welgene) and cultivated at 37°C in a humidified 5% CO2 incubator.

Cell Viability Assay

The cells were seeded into each well of a 96-well plate at a concentration of 1×10^4 cells per well. After 24 h or 48 h of incubation, the medium was replaced with fresh medium with various concentrations of CK for 48 h. Then, EZ-Cytox (DoGen) solution was added into the medium according to manufacturer's instructions and incubated at 37°C for 2 h. The optical density of each well was read with an ELISA reader (DiaTeK) at 450 nm. The percent cell viability was calculated as follows:

Cell viability (%) = (OD of Treatment / OD of Control) × 100 (1)

The half maximal inhibitory concentration (IC50) values were determined by plotting a linear regression curve.

Table 1: List of qPCR primer sequences

Primer name	Sequence (5' to 3')
BAK1-For	CCTCTGCAACCTAGCAGCACCA
BAK1-Rev	AGTTCAGGGCTGCCACCCAG
Caspase 3-For	CATAAAAGCACTGGAATGACAT
Caspase 3-Rev	CGCCAAGAATAATAACCAGG
Caspase 8-For	CACGACCTTTGAAGAGCTTC
Caspase 8-Rev	GAATATCATCGCCTCGAGG
Caspase 9-For	CAGAAAGACCATGGGTTTGA
Caspase 9-Rev	GAAACAGCATTAGCGACCC
Caspase 12-For	GAAGCCATGGCTGATGAGA
Caspase 12-Rev	CTGAACTCAGCTGTTTTTTGG
CD1-For	GCCCTCTGTGCCACAGATGTGA
CD1-Rev	CTTCTGGTATCAAAATGCTCCGGA
CDK4-For	GGCACTTACACCCGTGGTTGTTAC
CDK4-Rev	AGAGTGCTGCAGAGCTCGAAAGG
MMP2-For	CTTCTTCTTCAAGGACCGGTTCATT
MMP2-Rev	CTTGAAGAAGTAGCTGTGACCGCC
MMP9-For	GCTGTATTTGTTCAAGGATGGGAAGT
MMP9-Rev	GGCAGAAATAGGCTTTCTCTCGGT
mTOR-For	GGAAACATCCTTTAATCAGGCCTATG
mTOR-Rev	ACAGCGTATCTCTGGATGCTGAGG
P21-For	GGCAGACCAGCATGACAGATTTCT
P21-Rev	GGGTGAATTTCATAACCGCCTGT
P53-For	GTCTGTGACTTGCACGTACTCCCC
P53-Rev	CCGTCCCAGTAGATTACCACTGGA
Raptor-For	CACATCGTGAGTGTGAGCGTCAAT
Raptor-Rev	CTAAGGCAGCAGCCAACGTTCA
K-Ras-For	CATCAGCAAAGACAAGACAGGGTGT
K-Ras-Rev	CAGTTTCTTTTTCACAGGCATTGCT
H-Ras-For	GAATACGACCCCACTATAGAGGATTCC
H-Ras-Rev	TAGAAGGCATCCTCCACTCCCTG
N-Ras-For	TCAGCCAAGACCAGACAGGGTGT
N-Ras-Rev	CAGATGAAAAACCTGGGGTGGC
VEGF-For	CTACCTCCACCATGCCAAGTGGT
VEGF-Rev	CTCTCCTATGTGCTGGCCTTGGT
GAPDH-For	GTATCGTGGAAGGACTCATGACCAC
GAPDH-Rev	GCCAAATTCGTTGTCATACCAGGAA

Wound Healing Assay

The cells were seeded in 6-well plates at a concentration of 3×10^5 cells per well and incubated until they reach 80-90% confluence. A 100 µL pipette tip was used to make a single scratch in the cell monolayer and image of the scratch was captured at 0 h with a light microscope (magnification, ×10). Subsequently, cells were treated with various concentrations of CK or vehicle (control group) and incubated for 48 h. The width of the scratch was photographed and measured at 24 h and 48 h. Wound closure was calculated according to the following formula:

Wound closure=100 - [(scratch width at 24 or 48 h / scratch width at 0 h) x100] (2)

qPCR

The cells were seeded in T-25 flask at a concentration of 0.7×10^6 cells and incubated until they reach 70-80% confluence. Cells were treated with IC50 concentrations of CK

or vehicle (control group) and incubated for 48 h. The cells were washed with PBS and the cell pellet was harvested by centrifuging at 3,000 rpm for 2 min. Total RNAs were isolated using AccuPrep® Universal RNA extraction Kit (Bioneer) and cDNA synthesis using PrimeScript IITM RT Reagent Kit (TaKaRa bio).

Quantitative polymerase chain reaction (qPCR) was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) with gene-specific primers (Table 1) on a LightCycler 480 system (Roche) according to the manufacture's protocol. For analyzing the relative expression of each gene, GAPDH as a reference gene was used to normalize, and LC480Conversion and LinRegPCR software (Heart Failure Research Center) were used for analyzing the relative expression of each gene.

Statistical Analysis

The data were presented as the average data (Mean ± SE) obtained by repeating three or more times in each experiment. The data were analyzed and tested using a student t-test at $p<0.05$.

RESULTS

Inhibition of SK-N-MC Cell Growth and IC50 of CK

To investigate the proliferation inhibitory effect of CK on brain neuroblastoma cells and to check the IC50 value, SK-N-MC cells were treated with 0, 20, 40, 60, 80, and 100 μM of CK for 48 h and the cell viability was compared (Figure 1). The cell viabilities of all CK treated groups showed significant decreases compared to the untreated group (control, 0 μM). As the concentration of CK increased, the cell viability drastically decreased. In particular, CK treatment of 40 μM or greater induced rapid cell death by showing cell viability of 28.5% or less compared to the untreated group. And also, it was confirmed that IC50 concentration of CK equivalent to 50% neuroblastoma cell viability was 33.06 μM.

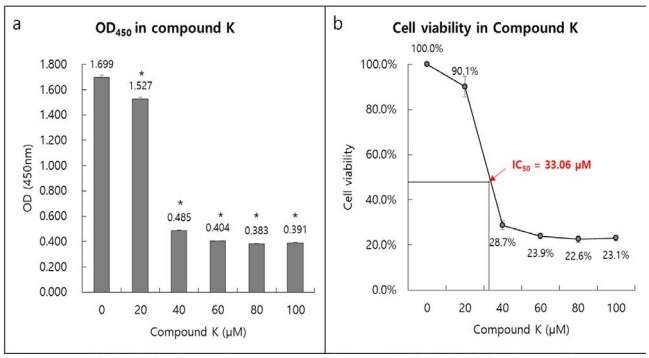

Figure 1: Cell viability of CK-treated cells and IC50 concentration of CK against neuroblastoma cells. (a) SK-N-MC cells were treated with 0, 20, 40, 80, 100 μM of CK and cultured at 37°C, 5% CO2 incubator for 48 h, and then the OD450 of the cells was measured (Mean ± SE, * $p < 0.01$). (b) Cell viability of each treatment group was expressed as a percentage based on the control group. The IC50 concentration of CK for 50% survival of neuroblastoma was determined by plotting a linear regression curve.

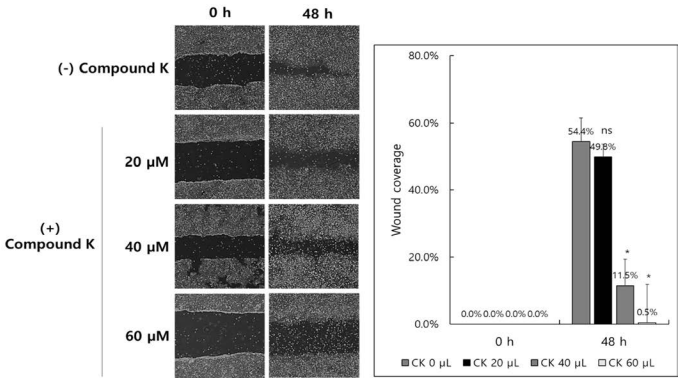

Figure 2: Scratch wound healing assay to confirm the effect of CK to inhibit migration of neuroblastoma cells. (Left) SK-N-MC cells were treated with 0, 20, 40, and 60 μM of CK for 48 h and images were recorded at 0 h and 48 h after scratching. (Right) The wound coverage % was determined by the aera of cells migrating towards the scratched area over time using ImageJ TM software (Mean ± SE, * $p < 0.05$).

Inhibition of SK-N-MC Cell Migration by CK

The process of tumor cell invasion and metastasis is understood as the migration of individual cells isolated from the primary tumor. In order to find out whether CK inhibits the migration of neuroblastoma, SK-N-MC cells proliferated in a culture dish treated with CK were scratched and the degree of cell migration was observed after 48 h (Figure 2). SK-N-MC cells were treated with 0, 20, 40, 60, 80, and 100 μM of CK, but the cells in the 80 and 100 μM CK treated groups did not grow much at all to give scratch, so they were excluded from the results in the Figure 2.

As the cells proliferated, the scratch area started to fill from the outside to the inside, and in the CK untreated group, most of the scratched area was covered with cells after 48 h, and it was found that cell migration proceeded very actively. The average cell migration rate of the untreated group analyzed by Image J program was 54.4%. The wound coverage rate of CK 20 μM treatment group was 49.8% on average, and there was no significant difference with the control group. In CK 40 μM and 60 μM treatment groups, the wound coverage was lowered to an average of 11.5 and 0.5%, respectively, showing a highly significant difference compared to the control group. These results demonstrated that the migration of neuroblastoma cells was strongly inhibited when treated with a CK IC50 of 33.06 μM and

higher, which was confirmed through a cell viability test.

Apoptosis and Anticancer Regulatory Gene Expression in Neuroblastoma cells by CK

In order to analyze the effect of CK on neuroblastoma cells leading to apoptosis, which are associated with cell cycle, metastasis, angiogenesis and etc., the expression of 17 related genes was confirmed through qPCR (Figure 3).

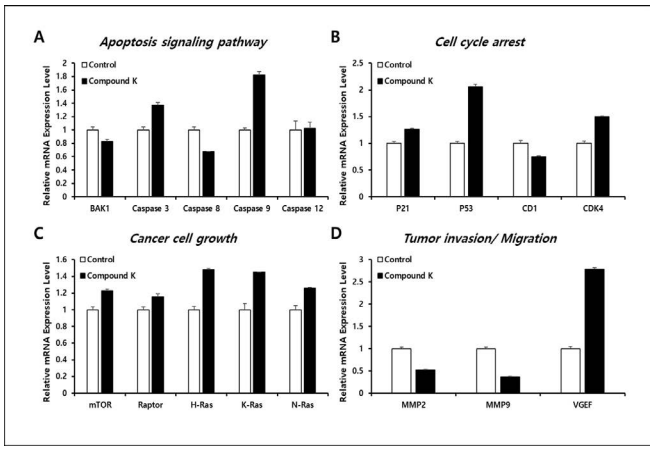

Figure 3: Expression of Apoptosis-regulatory genes after treatment with CK Relative expression of genes was determined by qPCR in SK-N-MC cells treated with or without CK of the concentration of IC50 for 24 h. Result means (±SE) of three biological replicates. All protein expressions were significantly different (at p=0.05) except Caspase 12.

By analyzing BAK1, caspase -3, -8, -9, -12 genes in the apoptosis signaling pathways, the expression of caspase 3 and caspase 9 was increased. This indicates that apoptotic signaling of caspase 9 of the intrinsic pathway and caspase 3, a downstream effector, was activated by CK through which apoptosis was executed by cleaving targeted cellular proteins (Galluzzi et al., 2018). However, the decreased expression of BAK1, a pro-apoptotic gene of the mitochondrial pathway, suggests that BAK1 is not a direct cause of caspase 9 activity. In addition, the expression of caspase 8 was also reduced, suggesting that CK-induced apoptosis of neuroblastoma cells was not due to activation of an extrinsic apoptotic pathway.

The oncogenes, p53 and p21, are related tumor suppressor proteins and cyclin-dependent kinase (CDK) inhibitors, respectively, and these proteins form a complex with each other to prevent cancer metastasis and recurrence (Gartel et al., 1996; Gartel and Tyner, 1998; Jeong, 2017). Both p53 and p21 expression were increased in CK-treated neuroblastoma cells, and in particular, the expression of p53 was greatly increased.

In the cell cycle-related enzyme genes, CD1 (cyclin D1) and CDK4 (cyclin dependent kinase 4), the expression of CD1 was decreased and the expression of CDK4 was increased. This means that CK acts as an inhibitor of CDK4/CD1 activity by interfering with the binding of CD1 to CDK in the G1 phase of the cell cycle and blocking the progression to the G2 phase (Kim et al., 2000).

mTOR (rapamycin mammalian target) is a gene that regulates cell growth and proliferation, autophagy and protein synthesis (Fingar and Blenis, 2004). Inhibition of mTOR is known to exhibit anticancer effects such as inhibition of G1-S phase conversion during cell division and inhibition of angiogenesis (Shin et al., 2011), but CK treatment did not inhibit mTOR expression in neuroblastoma cells. Moreover, the expression of the Raptor (regulation-related protein of mTOR) gene essential for mTOR signaling was also slightly increased in the CK-treated group.

Ras, the oncogene, also plays a central role in cell signaling pathways. It is well known that increased expression of Ras in normal cells induces transformation into cancer cells, but in vitro, expression of about 100-fold or more is required, and transformation is not induced below the threshold (Hua et al., 1997). In this experiment, the expression of structurally and functionally similar H-, K-, and N-Ras genes was observed. Although the expression of all three genes was slightly increased in the CK-treated group, it is considered not to a level that promotes the proliferation of cancer cells.

Tumor invasive growth or metastasis is closely related to proteolytic enzymes MMPs (matrix metalloproteinases), of which MMP2 and MMP9 play an important role in the degradation of basement membrane and extracellular matrix (Hanemaaijer et al., 2000). It is known that when the expression of MMP2 and MMP9 in cancer cells is high, metastasis is frequent and the prognosis is poor (Talyensaari et al., 2003; Pacheco et al., 1998). However, the expression of these genes was strongly inhibited by CK treatment. All tests were made by a student t-test between control and treated groups at p=0.05 in Figure 3.

VEGF (vascular endothelial growth factor) induces the generation and migration of vascular endothelial cells, and the endothelial cells induce tumor growth by producing tumor growth factors (Jeong, 2001). The expression of VGEF was significantly increased in the CK-treated group, indicating that there surprisingly was no inhibition of angiogenesis.

DISCUSSION

Ginsenoside CK has been extensively studied to have anticancer effects on various carcinomas, but its anticancer

effects on neuroblastomas have not much been reported. In this study, to investigate whether CK is effective as a new anticancer agent for neuroblastoma with poor prognosis, cell survival and cell migration were analyzed. In addition, the expression of 17 genes in signal transduction pathways related to cancer cell death, metastasis, cell cycle and angiogenesis were analyzed.

The IC50 value of CK for neuroblastoma cells has not been reported so far, but the IC50 values for other carcinoma cell lines such as HL-60, U937, and HeLa have been reported as 14.1-59.4 μM (Cho et al., 2009). In this experiment, the IC50 value of CK for SK-N-MC cells was also 33.06 μM, which was similar to the values for other cancer cell lines.

Inhibition of cell proliferation is closely related to cell cycle arrest. From the result of analyzing the expression of the cell cycle-related genes CD1, CDK4, mTOR, Raptor, H-, K- and N-Ras, CD1 expression was reduced among them, indicating that G1-G2 phase conversion of the cell cycle was blocked (Fingar and Blenis, 2004). However, the expressions of the remaining genes were somewhat increased, assuming that blocking at other locations in the cell cycle was not possible.

CK treatment at 40 μM and 60 μM, higher than the IC50, lowered the wound coverage rate of SK-N-MC cells to 11.5% and 0.5%, and strongly inhibited cell migration. Inhibition of cell migration is closely related to metastasis of cancer cells, and the expression of related genes p53 and p21 significantly increased in the CK-treated group, supporting this cell migration suppression result. Moreover, the expression of MMP2 and MMP9, which promote tumor invasive growth or metastasis, was also strongly suppressed.

Apoptotic morphological changes were observed in most SK-N-MC cells treated with CK at IC50 concentration. In this regard, as a result of observing the expression of BAK1, caspase -3, -8, -9, and -12 on the apoptosis signaling pathway, the expressions of caspase 9 and caspase 3 were increased, but the expression of caspase 8 was decreased, so that the intrinsic signaling pathway was activated and it was confirmed that apoptosis had progressed.

VEGF and mTOR genes promote cancer proliferation by inducing angiogenesis around cancer cells (Shin et al., 2011; Jeong, 2001), but expressions of both genes were not suppressed in the CK-treated group, but rather increased.
In conclusion, ginsenoside Compound K showed excellent anticancer activity against neuroblastoma cells, such as inhibitor of CDK4/CD1, inhibition of cell proliferation, metastasis and migration by tumor suppressor genes, and induction of intrinsic signaling apoptosis pathway. Therefore, CK proved to be a novel anticancer candidate for the treatment of neuroblastoma. The effect of CK on neuroblastoma was quite significant in this study but less attention has been focused on anticancer effect in brain cancer. Though CK has been much published and is well known for its anticancer effects in various cancers. Further studies are needed in detecting various cancer cell signalings as well as investigating different cancer microenvironments.

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CONFLICT OF INTEREST

There is no conflict of interest.

NOVELTY STATEMENT

Ginsenoside compound K showed excellent anticancer activity against neuroblastoma cells, such as inhibitor of CDK4/CD1, inhibition of cell proliferation, metastasis and migration.

AUTHORS CONTRIBUTION

Seun Eui Kim contributed a major work and others equally contributed. Dr. Gwang Joo Jeon has mainly designed and guided the experiment.

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