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Research article

Korean Red Ginseng extract reduces hypoxia-induced epithelial-mesenchymal transition by repressing NF- κ B and ERK1/2 pathways in colon cancer



Eui Joo Kim¹, Kwang An Kwon^{1,2}, Young Eun Lee¹, Ju Hyun Kim¹, Se-Hee Kim^{2,**},
Jung Ho Kim^{1,2,*}

¹ Division of Gastroenterology, Department of Internal Medicine, Gachon University Gil Medical Center, Gachon University School of Medicine, Incheon, Republic of Korea

² Gachon Medical Research Institute, Gachon University Gil Medical Center, Incheon, Republic of Korea

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ABSTRACT

Background: The incidence of colorectal cancer (CRC) is increasing, with metastasis of newly diagnosed CRC reported in a large proportion of patients. However, the effect of Korean Red Ginseng extracts (KRGE) on epithelial to mesenchymal transition (EMT) in CRC is unknown. Therefore, we examined the mechanisms by which KRGE regulates EMT of CRC in hypoxic conditions.

Methods: Human CRC cell lines HT29 and HCT116 were incubated under hypoxic (1% oxygen) and normoxic (21% oxygen) conditions. Western blot analysis and real-time PCR were used to evaluate the expression of EMT markers in the presence of KRGE. Furthermore, we performed scratched wound healing, transwell migration, and invasion assays to monitor whether KRGE affects migratory and invasive abilities of CRC cells under hypoxic conditions.

Results: KRGE-treated HT29 and HCT116 cells displayed attenuated vascular endothelial growth factor (VEGF) mRNA levels and hypoxia-inducible factor-1 α (HIF-1 α) protein expression under hypoxic conditions. KRGE repressed Snail, Slug, and Twist mRNA expression and integrin α V β 6 protein levels. Furthermore, hypoxia-repressed E-cadherin was restored in KRGE-treated cells; KRGE blocked the invasion and migration of colon cancer cells by repressing NF- κ B and ERK1/2 pathways in hypoxia.

Conclusions: KRGE inhibits hypoxia-induced EMT by repressing NF- κ B and ERK1/2 pathways in colon cancer cells.

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1. Introduction

Colorectal cancer (CRC) is the second most commonly diagnosed cancer in men and the third in women in South Korea [1]. Moreover, CRC incidence in South Korea is increasing at a rate of approximately 6% per year [1]. Notably, metastatic status, including locoregional node-positive at newly diagnosed CRC, has been reported in 57% of CRC patients [2].

Due to high oxygen requirements of rapid cell proliferation, solid tumors, such as CRC, often contain hypoxic regions and structurally and functionally unusual intratumoral blood vessels

[3]. Intratumoral hypoxia induces the accumulation of hypoxia-inducible factor-1 α (HIF-1 α), a protein that is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions [3,4]. HIF-1 α plays a key role in tumor progression, therapeutic resistance, invasiveness, and metastasis [5–7].

In the initial stages of metastasis, cancer cells separate from the main tumor sites, migrate, and invade the surrounding tissue, i.e. lymphatic and blood vessels. During the epithelial-mesenchymal transition (EMT), epithelial cells lose their cell-cell junctions and polarity, acquiring migratory and invasive abilities and displaying mesenchymal cell phenotype [8]. EMT is an important cellular

* Corresponding author. Division of Gastroenterology, Department of Internal Medicine, Gachon University Gil Medical Center, Gachon University School of Medicine, Namdong-daero 774, Namdong-gu, Incheon 21565, South Korea.

** Corresponding author. Gachon Medical Research Institute, Gachon University Gil Medical Center, 21 Namdong-daero 774 Beon-gil, Namdong-gu, Incheon 21565, Republic of Korea.

E-mail addresses: sehee0423@gilhospital.com (S.-H. Kim), junghokimm@gilhospital.com (J.H. Kim).

event that enables malignant cells in the primary tumor to invade other tissues and metastasize [8,9]. Importantly, EMT is mainly triggered by tumor hypoxia [7,8].

Korean Red Ginseng (RG) (*Panax ginseng* Meyer) is commonly used in Asian traditional medicine to treat various diseases [10]. Two-thirds of cancer patients in Korea take dietary supplements; of these, 50% have reported taking an RG product [11,12]. Korean RG extract (KRGE) has long been used in tonics and rejuvenation remedies [13]. Although beneficial anti-cancer activity of KRGE has been reported in vitro and in vivo, detailed molecular mechanisms of the anti-tumor effects are not well understood [14–16].

Although a few studies have reported detailed molecular mechanisms of anti-metastatic effects of KRGE in CRC, the effects of KRGE on the EMT process in CRC metastasis are unknown. Therefore, the aim of this study was to evaluate the effects of KRGE on hypoxia-induced EMT in CRC cell lines.

2. Materials and methods

2.1. Reagents

KRGE was manufactured by Korea Ginseng Corporation (Seoul, South Korea) from six-year-old Korean RG plants (*P. ginseng*). The roots of Korean RG were extracted by steaming fresh Korean Ginseng harvested in South Korea at 90–100°C for 3 h and then drying the plant material at 50–80°C. KRGE was extracted at 85–90°C for 8 h by circulating hot water through it three times. The water content of the collected extract was 36% of the total weight. MG132 and deferoxamine (DFO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). DMSO was used to dissolve MG132. KRGE and DFO were dissolved in water.

2.2. Cell lines and hypoxic conditions

HT29 and HCT116 human colon cancer cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). The cells were cultured in McCoy's 5A medium (Gibco, Carlsbad, CA, USA) with 1% penicillin streptomycin (Gibco) and 10% fetal bovine serum (FBS; Gibco), at 37°C in a 5% CO₂ humidified incubator. To generate hypoxic conditions, the cells were incubated in a hypoxic incubator (New Brunswick Scientific, Edison, NJ, USA) with 1% O₂ and 5% CO₂ balanced with 94% N₂.

2.3. MTT cell proliferation assay

KRGE-treated cells were incubated in 96-well plates for 24–96 h. The MTT reagent (Sigma-Aldrich) (5 mg/mL) was diluted in McCoy's medium. After 90 min of incubation, the medium was replaced by 100 µL of DMSO. Absorbance was measured at 570 nm.

The IC₅₀ values of KRGE were derived from dose-response curves using GraphPad Prism 3.05 (San Diego, CA, USA).

2.4. Western blot analysis

For cell lysis, RIPA buffer (50mM Tris-HCl at pH 8.0; 0.1% SDS; 0.5% sodium deoxycholate; 150mM NaCl; 1% NP-40; protease inhibitors) and whole cell lysate buffer (0.1mM EDTA; 400mM NaCl; 10mM HEPES at pH 7.9; 0.1mM EDTA; 1mM DTT; 5% glycerol; protease inhibitors) were used. Nuclear and cytoplasmic fractions were extracted with the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific, Rockford, IL, USA). Anti-HIF-1 α antibody (1:1000) and anti-integrin α V β 6 antibody (1:1000) were obtained from Novus Biologicals (Littleton, CO, USA). Antibodies to E-cadherin (1:5000), phospho-p65 (1:1000), p65 (1:1000), phospho-ERK1/2 (1:1000), ERK1/2 (1:1000), phospho-p38 (1:1000), p38 (1:1000), phospho-JNK (1:1000), JNK (1:1000), phospho-STAT3 (1:1000), STAT3 (1:1000), histone H3 (1:1000), and GAPDH (1:1000) were purchased from Cell Signaling (Beverly, MA, USA). Anti- β -actin antibody (1:5000) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The samples were normalized to β -actin using Image J software.

2.5. Quantitative real-time PCR (qRT-PCR)

QRT-PCR was conducted on a CFX96 detection system (BioRad, Hercules, CA, USA) with SYBR Green as the marker (Takara Bio Inc., Otsu, Shiga, Japan). Primers for the qRT-PCR reaction are listed in Table 1. The fold change in expression of target gene was calculated using GAPDH as reference. The experiment was performed in triplicate.

2.6. Immunocytochemistry

Cells treated with KRGE for 5–7 days were plated on coverslips coated with 0.1% gelatin [17]. After fixing in 3.7% paraformaldehyde for 15 min, the cancer cells were permeabilized in 0.5% Triton X-100/PBS for 5 min, and blocked in 1% BSA/PBS-T (0.1% Triton X-100/PBS) for 30 min. After incubation with anti-E-cadherin antibody diluted in 1% BSA/PBS-T for 1 h at room temperature, the cells were washed with PBS and incubated with Alexa Fluor 555 (Life Technologies, Carlsbad, CA, USA) for 1 h at room temperature. Cell nuclei were stained with DAPI (Vector Laboratories Inc., Burlingame, CA, USA) and the images were processed using a LSM710 confocal microscope (Carl Zeiss, Jena, Germany).

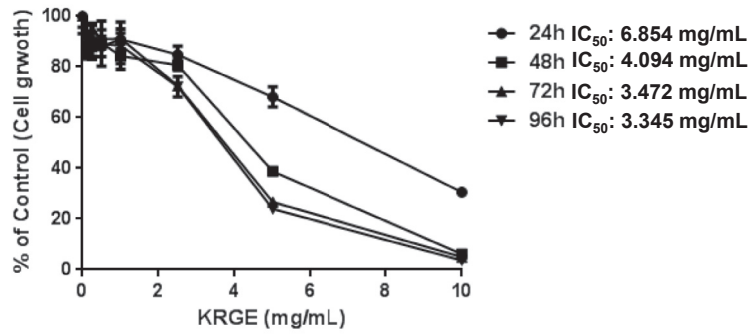
2.7. Scratched wound healing assay

Cells were cultured to 80–90% confluence in 60 mm plates and wounded using a tip. Afterwards, the cells were washed with PBS to remove non-adherent cells and incubated in McCoy's medium with

Table 1
Nucleotide sequences of qRT-PCR primers

Targets of PCR	Forward primers (5'–3')	Reverse primer (5'–3')
Snail	CCC CAACTCG GAA GCC TAA CT	GCT GGA AGG TAA ACT CTG GAT TAG A
Slug	ACG CCC AGC TAC CCA ATG	CGC CCC AAA GAT GAG GAG TA
Twist	GCG CTG CCG AAG ATC ATC	GGT CTG AAT CTT GCT CAG CTT GT
VEGF	ATC TTC AAG CCA TCC TGT GTG C	CAA GGC CCA CAG GGA TTT TC
PHD1	AGG CTG TCG AAG CAT TGG TG	GGG ATT GTC AAC GTG CCT TAC
PHD2	AAG CCC AGT TTG CTG ACA TT	TTA CCG ACC GAA TCT GAA GG
PHD3	AGC CCA TTT TTG CCA GAC TCC	GAT TTC AGA GCA CGG TCA GTC
VHL	GCA GGC GTC GAA GAG TAC G	CGG ACT GCG ATT GCA GAA GA
GAPDH	AGG TCG GAG TCA ACG GAT TTG G	ACA GTC TTC TGG GTG GCA GTG ATG

A HT29



B HCT116

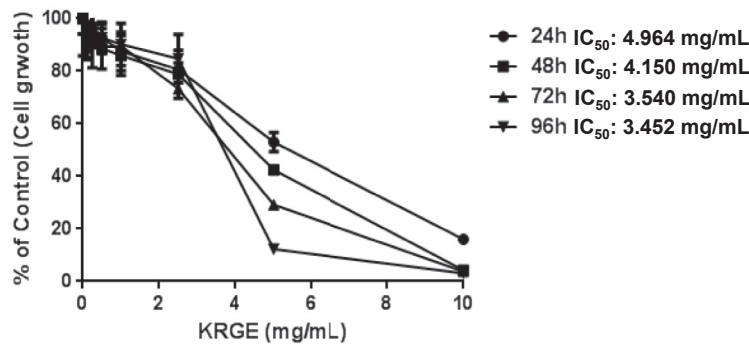
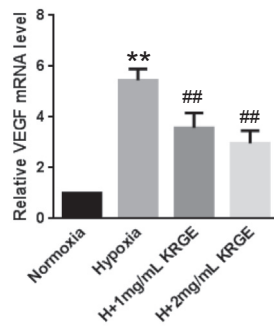
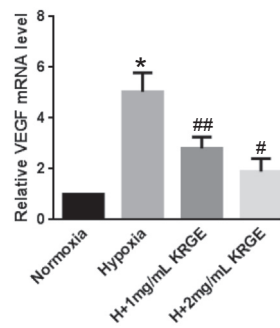


Fig. 1. Cytotoxic effects of KRGE on colon cancer cell lines. (A, B) Cell viability of HT29 and HCT116 cells treated with KRGE (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 mg/mL) for 24–96 h was estimated using the MTT assay. Data are represented as mean ± SD (n = 6). KRGE, Korean Red Ginseng extract.

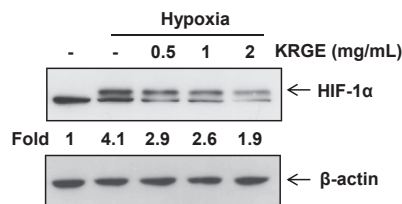
A HT29



B HCT116



C HT29



D HCT116

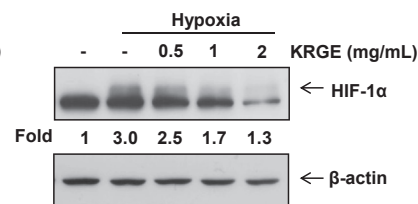


Fig. 2. Effects of KRGE on expression of VEGF mRNA and HIF-1 α protein levels in HT29 and HCT116 cells under hypoxic conditions. (A, B) Total RNA was isolated from cells treated with KRGE (1 and 2 mg/mL) and/or hypoxic incubation for 72 h (A) or 24 h (B). VEGF mRNA levels were analyzed by qRT-PCR. VEGF mRNA expression was reduced in HT29 and HCT116 cancer cells treated with KRGE under hypoxia. (C, D) HIF-1 α expression in cancer cells treated with KRGE (0.5, 1, and 2 mg/mL) and/or hypoxia for 24 h was examined using western blot. HIF-1 α protein expression was reduced in KRGE-treated HT29 and HCT116 cells under hypoxia. * p < 0.05, ** p < 0.01 versus normoxia control groups. # p < 0.05, ## p < 0.01 versus hypoxia-only groups. KRGE, Korean Red Ginseng extract; VEGF, vascular endothelial growth factor; HIF-1 α , hypoxia-inducible factor-1 α .

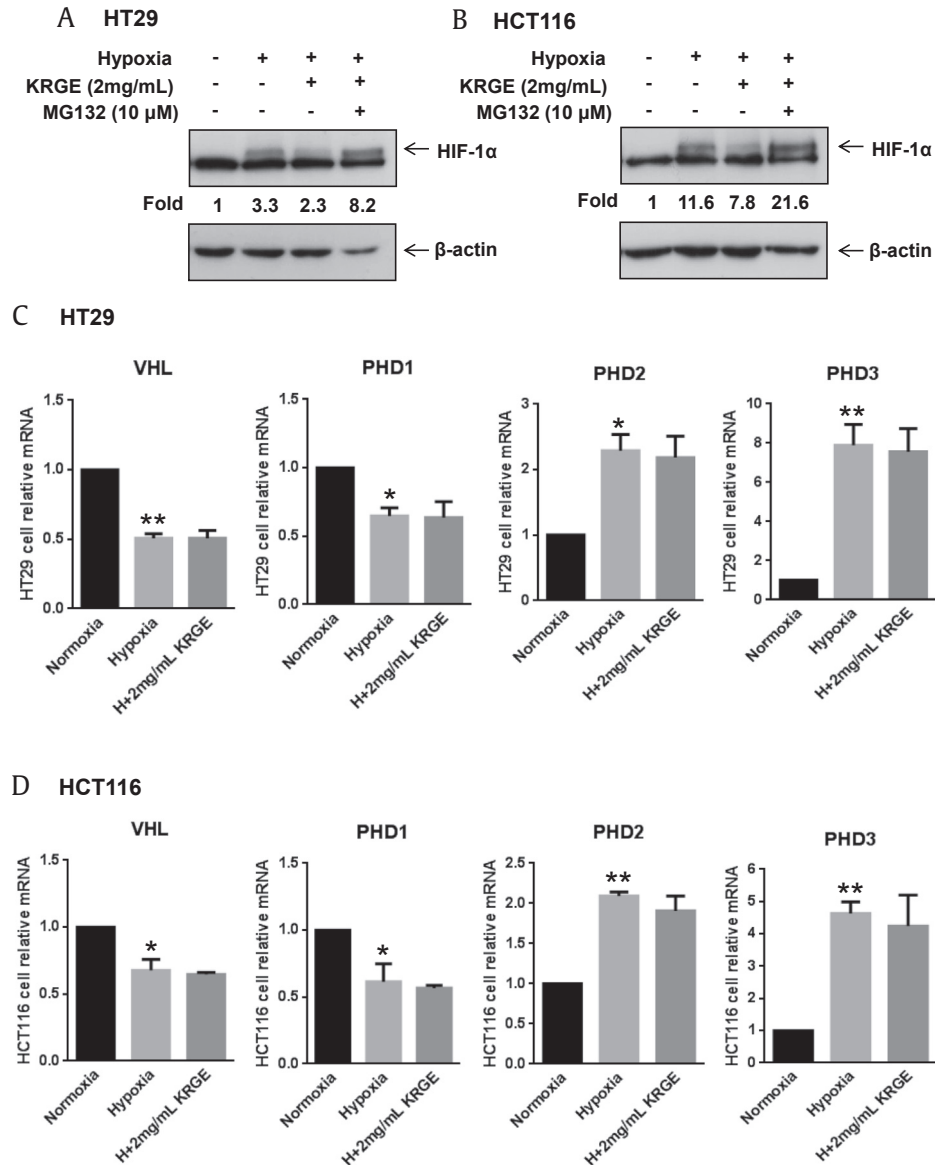


Fig. 3. Effects of KRGE on HIF-1 α degradation in hypoxic conditions. (A, B) HIF-1 α expression was examined in hypoxic cancer cells treated with 2 mg/mL KRGE after pretreatment with 10 μ M MG132 for 30 min. MG132 blocked KRGE-induced inhibition of HIF-1 α protein expression. (C, D) VHL, PHD1, PHD2, and PHD3 mRNA levels were analyzed by qRT-PCR and were unaffected in HT29 and HCT116 cancer cells treated with KRGE in hypoxic conditions. * $p < 0.05$, ** $p < 0.01$ versus normoxia control groups. KRGE, Korean Red Ginseng extract; HIF-1 α , hypoxia-inducible factor-1 α ; VHL, von Hippel-Lindau; PHDs, prolyl hydroxylases.

1–2 mg/mL KRGE and/or 100 μ M DFO for 48 h. Cell images were recorded at 0 and 48 h (CFX41, Olympus, Tokyo, Japan). The percentage of migrating cells was determined with Image J software as previously described [17]. The experiment was performed in triplicate.

2.8. Transwell migration assay

KRGE-treated cells were plated into transwells with 0.2% gelatin coated surface and exposed to hypoxia for 24 h [17]. FBS (20%) in the bottom chamber was used as a chemoattractant. After fixation with methanol for 2 min, the cells were stained with hematoxylin/eosin for 10 min. Thereafter, the cells on the upper membrane were removed with a wet cotton swab, whereas the cells on the lower membrane were mounted in Vectashield (Vector Laboratories,

Burlingame, CA, USA) and counted under a DP72 microscope (Olympus). The data were normalized to control group values. The experiment was performed in triplicate.

2.9. Matrigel invasion assay

After coating the lower surfaces with 0.2% gelatin for 1 h at room temperature and upper membranes with matrigel (BD Biosciences, San Jose, CA, USA) for 2 h at 37 $^{\circ}$ C, KRGE-treated cells in the transwells were subjected to hypoxia for 48 h, with 20% FBS in the bottom chamber used as chemoattractant. After fixation with methanol and staining with hematoxylin/eosin, the cells on the lower surfaces were counted under an Olympus DP72 microscope. The data were normalized to the control group. The experiment was performed in triplicate.

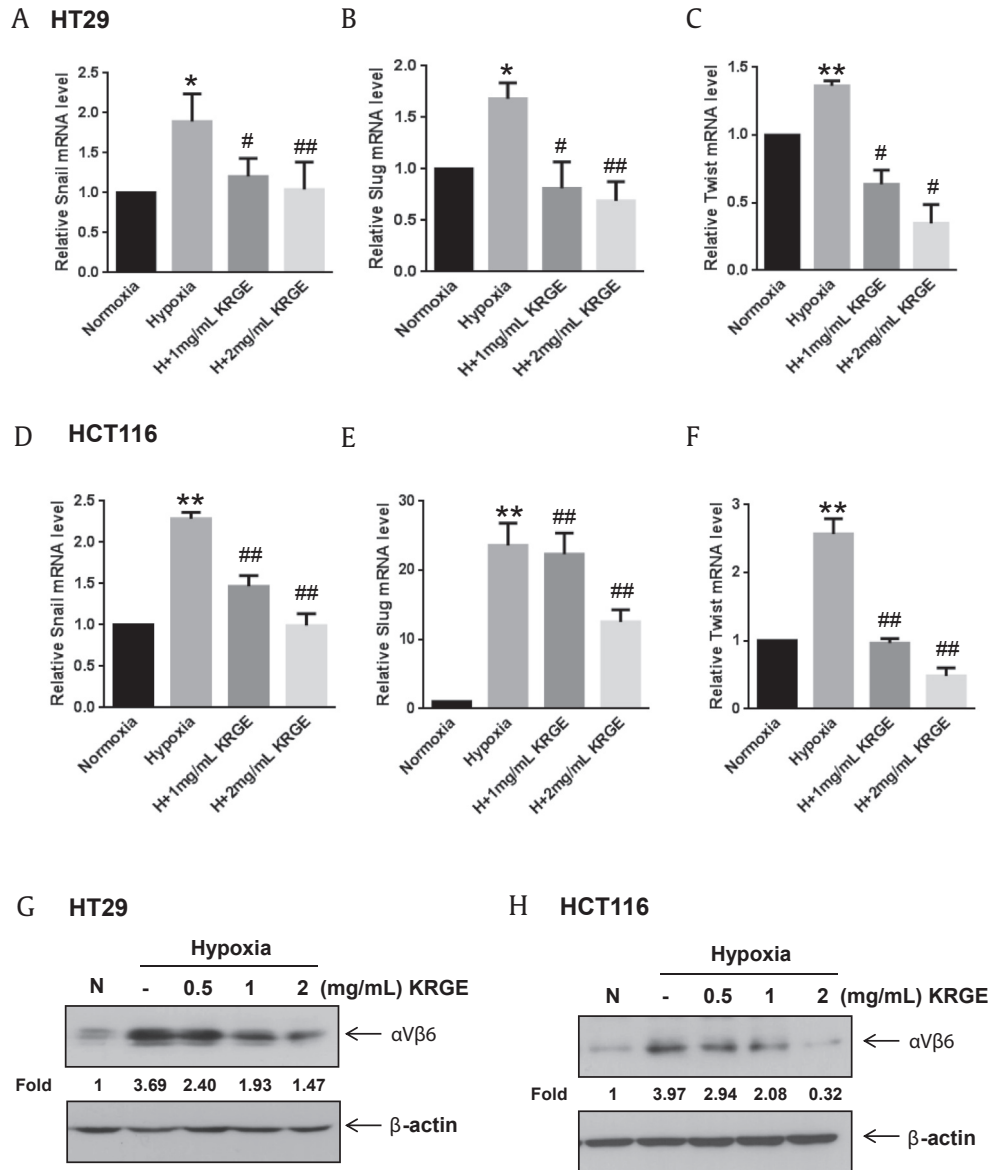


Fig. 4. Effects of KRGE on expression of EMT markers under hypoxic conditions. (A–F) Total RNA was isolated from cancer cells treated with KRGE (1 and 2 mg/mL) and/or hypoxic incubation for 72 (A–C) or 24 h (D–F). Transcripts of Snail (A, D), Slug (B, E), and Twist (C, F) were repressed in HT29 and HCT116 cancer cells treated with KRGE under hypoxic conditions. (G, H) α V β 6 integrin expression in cells treated with KRGE (0.5, 1, and 2 mg/mL) and/or hypoxic incubation for 7 (G) or 5 days (H) was examined by western blot. Fold changes of α V β 6 integrin expression were normalized to β -actin. α V β 6 integrin was repressed in KRGE-treated HT29 and HCT116 cells in hypoxic conditions. * $p < 0.05$, ** $p < 0.01$ versus normoxia control groups. # $p < 0.05$, ## $p < 0.01$ versus hypoxia-only groups. KRGE, Korean Red Ginseng extract; EMT, epithelial-mesenchymal transition; N, normoxia.

2.10. Statistical analysis

Data were analyzed with SPSS 23.0 software (IBM Corporation, Armonk, NY, USA). Significant differences between groups were assessed using a paired t-test. P-values < 0.05 were considered statistically significant.

3. Results

3.1. KRGE decreases VEGF mRNA levels and HIF-1 α protein expression

In order to examine cytotoxic effects of KRGE on colon cancer cells, HT29 and HCT116 cells were incubated with KRGE at various concentrations for 24–96 h, and cell viability was determined using the MTT assay (Fig. 1). As shown in Fig. 1, changes in IC_{50} values over

24–96 h were detected in both cell lines. In order to determine whether KRGE regulates the expression of hypoxia-related genes, HT29 and HCT116 cells were treated with 0.5–2 mg/mL KRGE and incubated under hypoxia for 72 and 24 h, respectively. As shown in Fig. 2A, KRGE-treated HT29 cells under hypoxia showed a 35–46% reduction in VEGF mRNA levels compared to the hypoxia control ($p < 0.01$). Similarly, KRGE-treated HCT116 cells under hypoxia exhibited a 44–62% decrease in VEGF mRNA expression compared to the hypoxia control ($p < 0.01$ and $p < 0.05$) (Fig. 2B). Furthermore, KRGE-treated HT29 and HCT116 cells displayed 29–51% and 17–57% decrease in HIF-1 α protein expression compared to hypoxia controls, respectively (Fig. 2C–D). As HIF-1 α protein is degraded via the ubiquitin-proteasome pathway, proteasome inhibitor MG132 was employed to investigate whether KRGE could regulate proteasomal degradation of HIF-1 α . Pretreatment with 10 μ M MG132 for 30 min under hypoxia blocked HIF-1 α

degradation in both cell lines treated with KRGE (Fig. 3A–B), suggesting that KRGE may reduce HIF-1 α stability in a proteasome-dependent manner.

Finally, in order to elucidate whether KRGE regulated the expression of von Hippel-Lindau (VHL) protein and prolyl hydroxylases (PHDs), which mediate HIF-1 α degradation, mRNA levels of VHL and PHDs in KRGE-treated cells were examined using qRT-PCR. No statistically significant change in VHL and PHDs mRNA levels was observed upon KRGE treatment in either of the cell lines, compared to hypoxia controls (Fig. 3C, D).

3.2. KRGE inhibits EMT markers expression under hypoxia

To determine whether KRGE could modulate EMT in hypoxic conditions, KRGE-treated HT29 and HCT116 cells were incubated under hypoxia for 72 or 24 h. Transcription of Snail, Slug, and Twist increased in hypoxic conditions and was decreased by KRGE treatment. Inhibitory effects of KRGE on mRNA levels of EMT markers in HT29 and HCT116 cells were as follows: in HT29 cells, Snail (36–46%, $p < 0.05$ and $p < 0.01$ versus hypoxia control groups), Slug (52–59%, $p < 0.05$ and $p < 0.01$ versus hypoxia control groups), and Twist (53–74%, both $p < 0.05$ versus hypoxia control groups); in HCT116 cells, Snail (36–57%, both $p < 0.01$ versus hypoxia control groups), Slug (5–47%, both $p < 0.01$ versus hypoxia control groups), and Twist (63–82%, both $p < 0.01$ versus hypoxia control groups) (Fig. 4A–F).

The effect of KRGE on expression of α V β 6 integrin, a mesenchymal phenotype marker in colon cancer, was also explored. As

shown in Fig. 4G–H, α V β 6 integrin expression increased under hypoxia, whereas the expression of α V β 6 integrin in KRGE-treated HT29 and HT116 cells was inhibited by 60 and 92%, respectively.

3.3. KRGE reverts hypoxia-induced morphological changes

Next, we evaluated whether KRGE could restore morphological characteristics of cells altered by hypoxic treatment. In order to perform this experiment, KRGE-treated HT29 and HCT116 cells were incubated under hypoxia for 7 or 5 days. Unlike normoxic HT29 and HCT116 cells, which were round and clustered together, hypoxia-exposed cells were scattered and elongated (Fig. 5A, C). Importantly, treatment with KRGE partially restored cell morphology to one reminiscent of cells grown under normoxia. Moreover, we found that E-cadherin protein levels that had been inhibited by hypoxia, recovered by 33 and 26% in KRGE-treated HT29 and HCT116 cells, respectively (Fig. 5B, D).

3.4. KRGE diminishes invasiveness of colon cancer cells under hypoxia

The scratched wound healing assay was performed in HT26 and HCT116 cells in order to assess whether KRGE regulates migration of colorectal cancer cells. A hypoxia mimicking reagent, DFO, was used. As shown in Fig. 6, DFO-treated HT29 and HCT116 cells showed enhanced migration compared to untreated groups, whereas migration of cells treated with DFO and KRGE was reduced. Specifically, KRGE showed a 56–71% ($p < 0.05$ versus DFO-

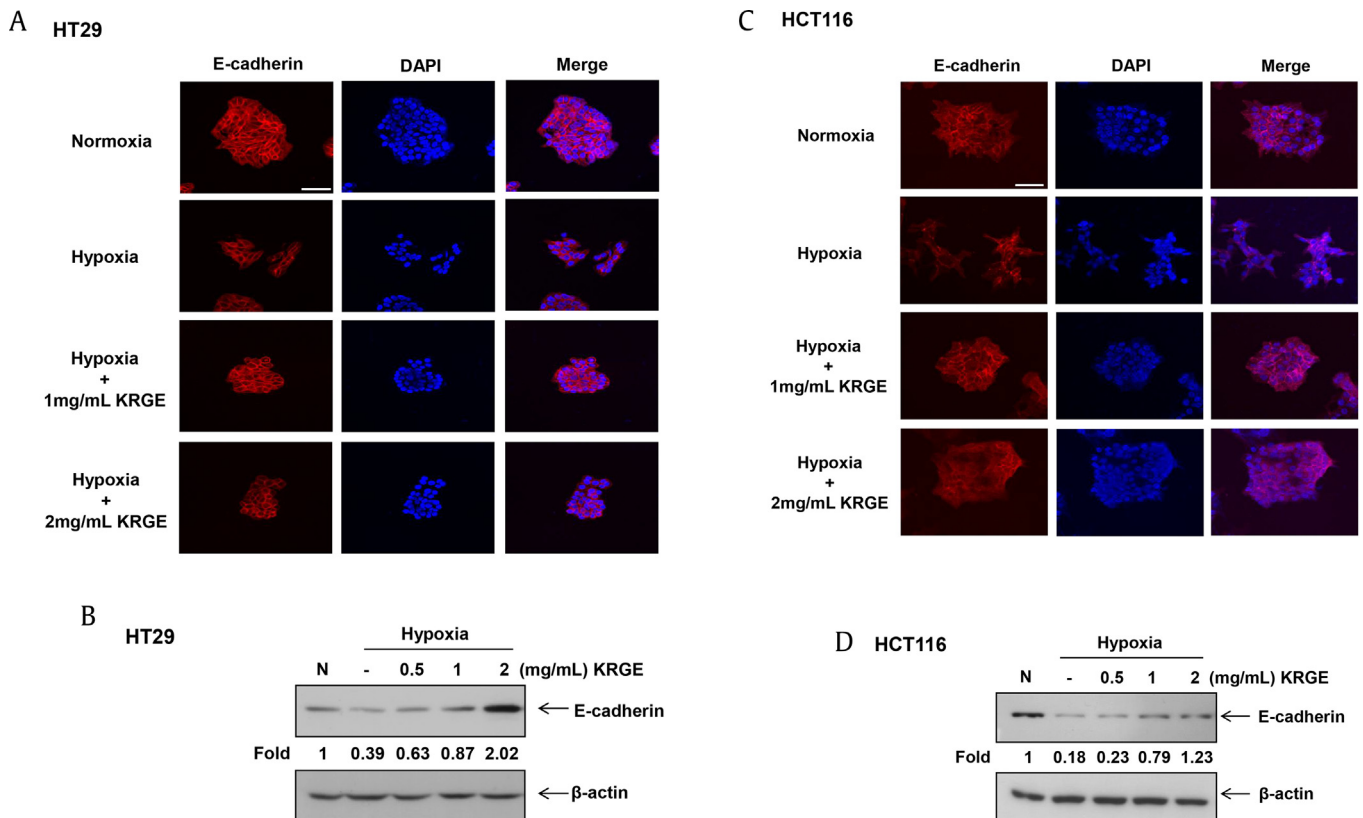


Fig. 5. Effects of KRGE on cell morphology and E-cadherin expression under hypoxia. (A, C) Immunohistochemical staining for E-cadherin expression and DAPI in KRGE-treated HT29 and HCT116 cancer cells in hypoxic conditions. DAPI (blue) and E-cadherin (red) were observed in cells treated with KRGE (1 and 2 mg/mL) and/or hypoxia for 7 (A, B) or 5 days (C, D). KRGE reversed morphological changes and E-cadherin levels of HT29 and HCT116 cells under hypoxia. (B, D) E-cadherin protein levels in cells treated with KRGE (0.5, 1, and 2 mg/mL) and/or hypoxia for 7 (B) or 5 days (D) were examined by western blot analysis. KRGE treatment restored E-cadherin levels in hypoxic HT29 and HCT116 cells. Magnification, 200 \times ; Scale bar, 50 μ m. KRGE, Korean Red Ginseng extract; N, normoxia.

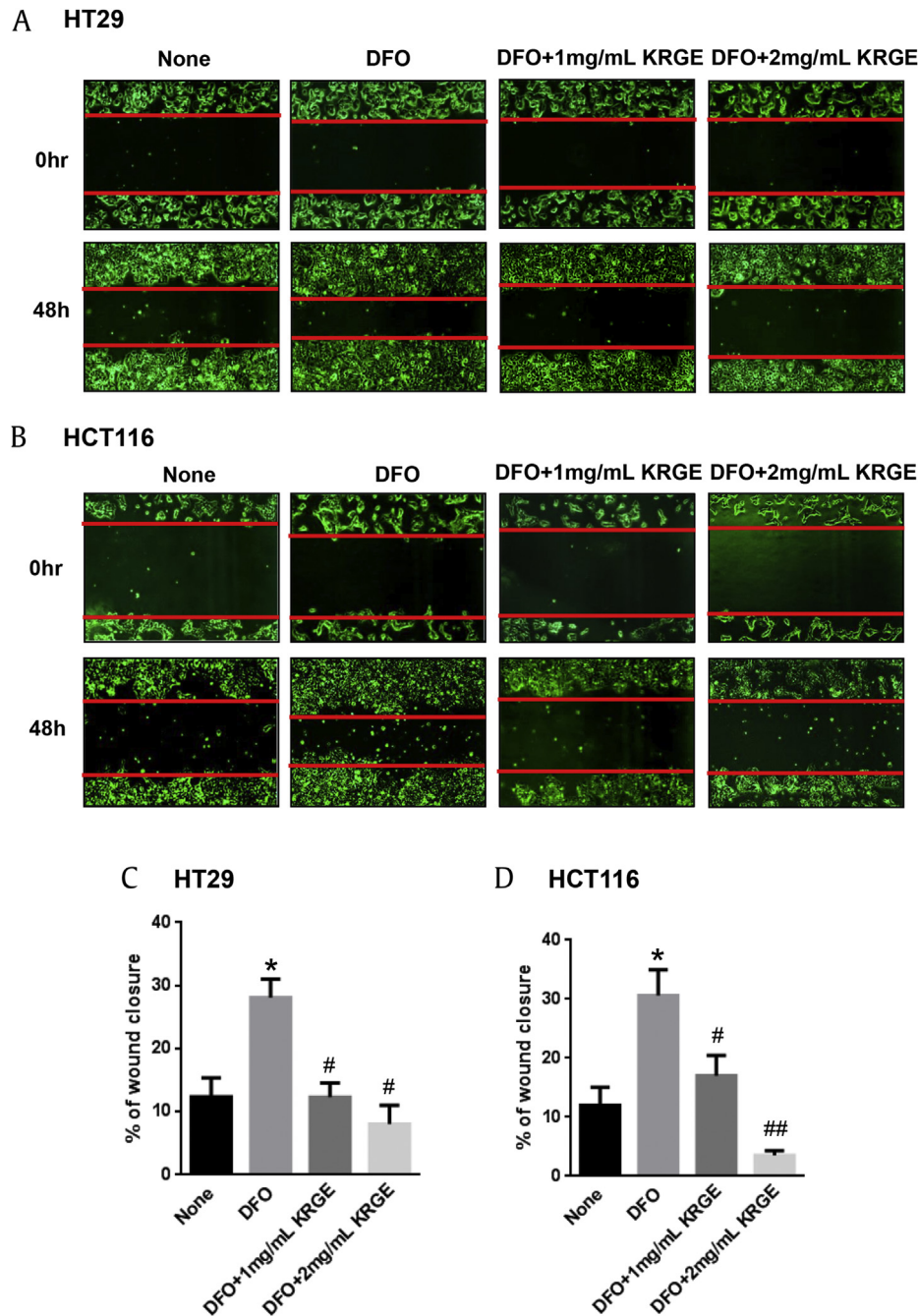


Fig. 6. Effects of KRGE on migratory ability of colon cancer cells under hypoxic conditions. (A, B) Scratched wound healing assay in HT29 and HCT116 cancer cells treated with KRGE (1 and 2 mg/mL) and/or DFO (100 μ M) for 24 h. (C, D) Effect of KRGE on migratory ability of HT29 and HCT116 cancer cells was quantified as a percentage of wound closure. KRGE-treated HT29 and HCT116 cancer cells showed reduced wound recovery in hypoxic conditions. Magnification, 40 \times . * p < 0.05 versus untreated control group. # p < 0.05, ## p < 0.01 versus DFO-only groups. KRGE, Korean Red Ginseng extract; DFO, deferoxamine.

only control groups) and 44–89% (p < 0.05 and p < 0.01 versus hypoxia control groups) decrease in wound recovery, in hypoxic HT29 and HCT116 cells, respectively. To confirm this suppressive effect of KRGE, we conducted the transwell migration assay. KRGE showed 38–78% (p < 0.05 versus hypoxia control groups) and 19–63% (p < 0.05 versus hypoxia control groups) decrease in migration of HT29 and HCT116 cells, respectively (Fig. 7A–B).

Finally, we performed the invasion assay to assess whether KRGE regulated the invasion of colon cancer cells. As shown in Fig. 7C–D, KRGE-treated HT29 and HCT116 cells displayed a 29–59% (p < 0.05 versus hypoxia control groups) and a 32–62%

(p < 0.05 versus hypoxia control groups) reduction of invasion under hypoxia, respectively.

3.5. KRGE represses the activation of NF- κ B and ERK1/2 under hypoxia

To identify cellular signaling pathways regulated by KRGE during the hypoxia-induced EMT, we examined various pathways in the KRGE-treated HT29 and HCT116 cells using western blot. KRGE-treated HT29 and HCT116 cells dramatically reduced hypoxia-induced phosphorylation of p65 and ERK1/2 (Fig. 8A–B). To

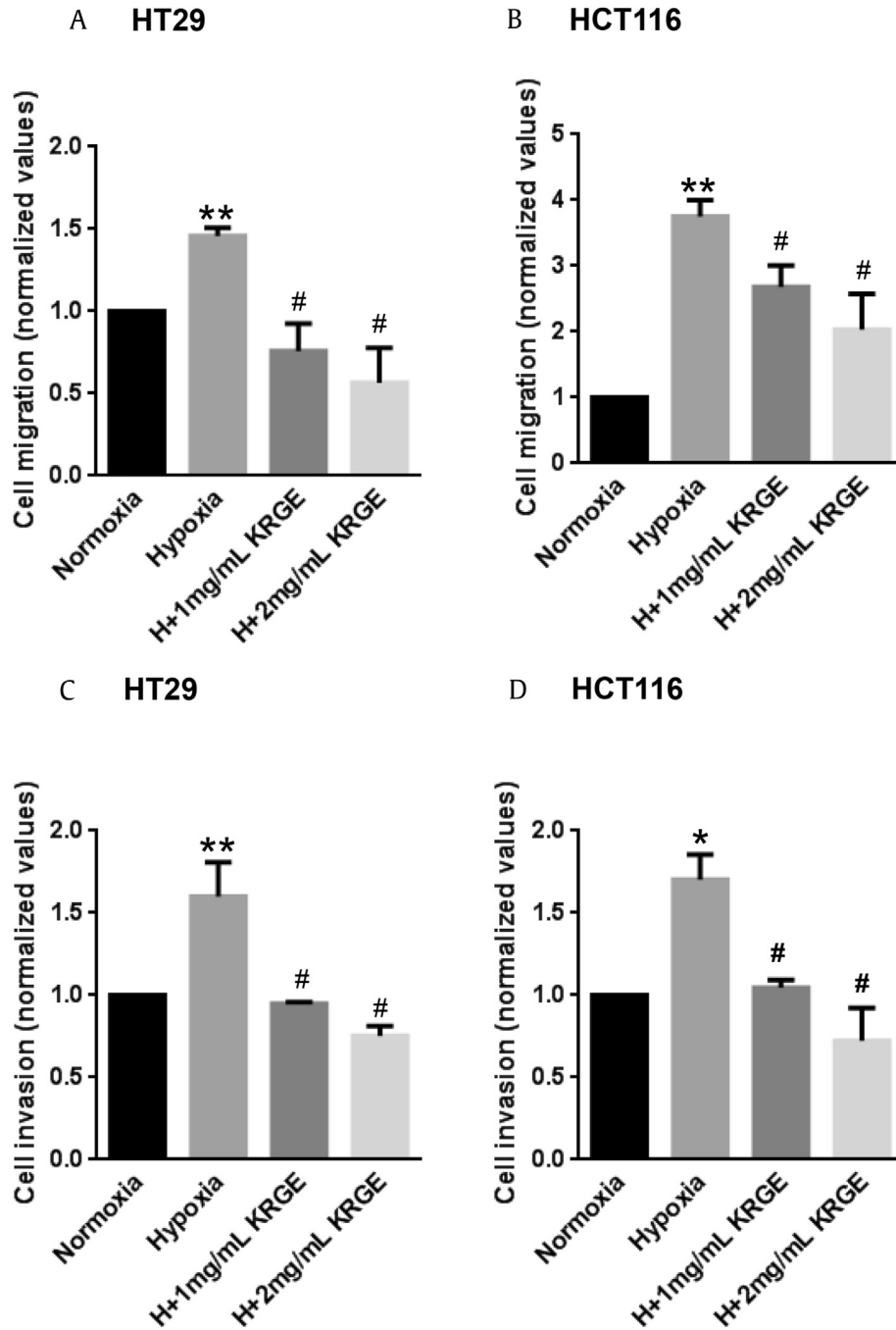


Fig. 7. Effects of KRGE on migratory and invasive properties of colon cancer cells under hypoxic conditions. (A–D) Transwell migration and matrigel invasion assay in HT29 and HCT116 cancer cells. KRGE-treated HT29 and HCT116 cancer cells were incubated for 48 (A, B) or 24 h (C, D) under normoxia or hypoxia. Cells on the upper surface of the transwells stained with H/E were counted and normalized to normoxia groups. KRGE inhibited migratory and invasive properties of HT 29 and HCT116 cancer cells in hypoxic conditions. * $p < 0.05$, ** $p < 0.01$ versus normoxia control groups. # $p < 0.05$ versus hypoxia-only group. KRGE, Korean Red Ginseng extract; H/E, hematoxylin and eosin.

confirm the inhibitory effect of KRGE on activation of p65, we examined cellular localization of p65 using nuclear and cytoplasmic fractions from KRGE-treated cells. As shown in Fig. 8C–D, KRGE inhibited the translocation of p65, but not STAT3.

4. Discussion

In cancer patients, metastases are among the main causes of death. Suppressing migratory and invasive abilities of cancer cells is especially important in colon cancer patients as distant metastasis is particularly common in CRC. Hypoxic control of EMT is notably

associated with cancer progression, metastasis, and resistance to therapy [5,18]. Moreover, HIF-1 α has been reported as an important regulator of EMT in several cancer cell lines [5,6,18].

In the current study, we hypothesized that KRGE suppresses migratory and invasive properties of colon cancer cells by repressing hypoxia-induced EMT. Our results indicate that KRGE inhibits hypoxic induction of VEGF by destabilizing HIF-1 α protein. HIF-1 α degradation results from proline hydroxylation by PHDs, which promotes binding to VHL protein, a component of the large E3 ubiquitin ligase complex, resulting in proteasomal degradation of HIF-1 α . In our study, KRGE did not reduce HIF-1 α stability in MG132-

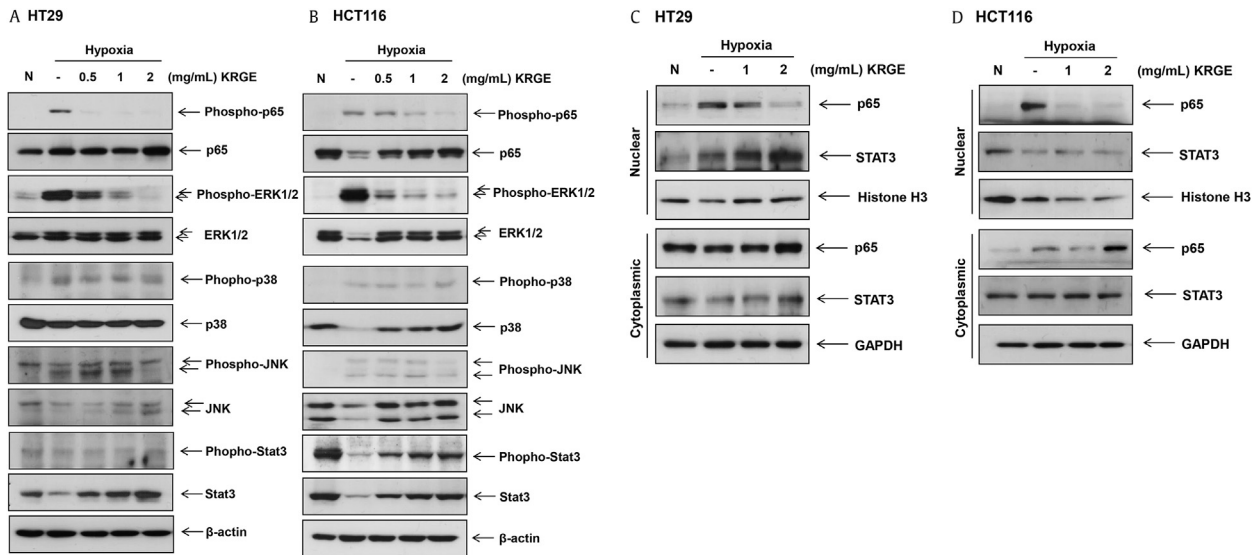


Fig. 8. Effects of KRGE on signaling pathways in hypoxia. (A–D) HT29 and HCT116 cells treated with KRGE (0.5–2 mg/mL) under hypoxia for 7 or 5 days were analyzed by western blot analysis. (A–B) Phosphorylation of p65 and ERK1/2 was decreased upon treatment with KRGE and hypoxia. (C–D) Nuclear translocation of p65 was inhibited in KRGE-treated cells. Histone H3 and GAPDH were used as loading controls of nuclear and cytoplasmic proteins, respectively. N, normoxia; KRGE, Korean Red Ginseng extract; Nuclear, nuclear extracts; Cytoplasmic, cytoplasmic extracts.

treated cells and did not affect mRNA levels of VHL and PHDs under hypoxia, indicating that KRGE may not control the expression of genes involved in HIF-1 α degradation, instead controlling the steps of the HIF-1 α degradation pathway, such as ubiquitylation. However, Choi et al. suggested that RG inhibits hypoxic induction of HIF-1 target genes through dissociation of HIF-1 dimer without affecting HIF-1 α stability in liver cancer and immortalized normal cells [14]. These conflicting results may result from organ specificity. Therefore, further studies are required to examine organ-specificity of KRGE-mediated regulation of HIF-1 α stability.

According to our results, KRGE treatment inhibited expression of EMT markers Snail, Slug, Twist, and α V β 6 integrin under hypoxic conditions. In addition, KRGE partially restored E-cadherin expression and reversed morphologic cell changes induced by hypoxic conditions. The process of EMT is involved in early embryogenesis, tissue fibrosis, and movement of metastatic cancer cells. Many of the molecules that trigger the EMT process have been identified, including Snail and Slug, transcription factors of target pathways that control EMT. In addition, Twist is upregulated during embryogenesis, tissue fibrosis, and cancer metastasis. Twist functions independently from Snail to suppress E-cadherin expression in metastatic cancer cells. Therefore, as the HT29 cell line displays low Twist mRNA levels, unlike HCT116, loss of E-cadherin in HT29 during hypoxia-induced EMT possibly occurs through Snail-dependent pathways.

Loss of E-cadherin expression is an important prototypical event in EMT [19]. In addition, colon cancer cells undergoing EMT show high levels of β 6 integrin [20]. For these reasons, we monitored E-cadherin and α V β 6 integrin expression levels in colon cancer cells. Incubation of cells for 5–7 days under hypoxia was required to detect morphological changes associated with the EMT process. KRGE increased E-cadherin in cancer cells under hypoxic conditions, and decreased α V β 6 integrin levels.

NF- κ B is a regulator of apoptosis, proliferation, angiogenesis, and metastasis [21,22]. Recent studies suggest that hypoxia induces NF- κ B activity in IKK- and TAK1-dependent manner [21], and activates the MAPK/ERK1/2 pathway that induces EMT-like phenotypes in hepatocellular carcinoma cells [23,24]. In this study, KRGE repressed hypoxia-induced phosphorylation of NF- κ B and ERK1/2 in colon cancer cells, indicating that KRGE may block metastasis of

colon cancer cells through inhibition of NF- κ B and ERK1/2. However, RG was reported to decrease metastasis of colon cancer cells by attenuating MMP-2 and MMP-9 pathways [25]. Therefore, RG may reduce invasion and metastasis of colon cancer cells via NF- κ B and ERK1/2 pathways in hypoxic conditions and MMP-2/9 pathways in normoxic conditions.

CRC is associated with two major classes of genetic instability, chromosome instability (microsatellite stable; MSS) and microsatellite instability (MSI) [26]. HT29 and HCT116 cancer cell lines were used in the present study as they belong to MSS and MSI cell lines, respectively. Our study of KRGE anti-metastatic effects in two different genetic signatures provides guidance for in vivo studies and clinical trials addressing KRGE-related molecular mechanisms.

Toxicity to healthy cells often limits the clinical utility of chemotherapeutic agents. As natural products, such as ginseng extracts, may present anti-cancer activity with reduced toxicity, it is important to elucidate the anti-cancer effects of KRGE.

In conclusion, KRGE inhibits hypoxia-induced EMT by repressing the activation of NF- κ B and ERK1/2 pathways in colon cancer cells and may be potentially beneficial in the treatment of colon cancer.

Conflicts of interest

The authors have no conflicts of interests to disclose.

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