**INTRODUCTION**

Recent research reports, based on the analysis of morbidity and mortality databases from 39 countries, indicated that the incidence of colorectal cancer continues to increase in young people in countries with a high human development index (Wong et al., 2021). Republic of Korea had the second highest incidence of colorectal cancer in 2018 (Khil et al., 2021).

Even after surgical intervention, some microscopic tumor cells may remain, which have the potential to invade the surrounding organs. Radiation therapy is commonly used to eliminate the remaining cells, which is termed adjuvant therapy (Baskar et al., 2012). Unfortunately, many clinical cases are associated with the development of radioresistance, which is difficult to overcome (Jin et al., 2016). Consequently, there is a great need for new chemotherapeutic agents with high anticancer activity to eradicate radioresistant cancer cells.

Recently, the scientific community has started to pay more attention to natural bioactive compounds and their anticancer properties. Shikonin is one of these naturally occurring compounds. Shikonin is extracted from *Lithospermum erythrorhizon*, *Onosma paniculata*, and *Arnebia euchroma*. Shikonin exerts cytotoxic effects by inhibiting protein tyrosine kinase and DNA topoisomerases, which play a crucial role in cancer cell DNA regulation, and decreases the expression of tumor necrosis factor receptor-associated protein 1. In addition, shikonin activated mitogen-activated protein kinases, and their specific inhibitors reduced the cytotoxic effects of shikonin. Additionally, shikonin decreased the migration of SNU-CSRR cells via the upregulation of E-cadherin and downregulation of N-cadherin. Taken together, these results suggest that shikonin induces mitochondria-mediated apoptosis and attenuates epithelial-mesenchymal transition in SNU-CSRR cells.

**Key Words:** Shikonin, Radiation resistance, Human colon cancer, Epithelial to mesenchymal transition, Mitochondria-mediated apoptosis, Mitogen-activated protein kinases

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**Natural Compound Shikonin Induces Apoptosis and Attenuates Epithelial to Mesenchymal Transition in Radiation-Resistant Human Colon Cancer Cells**

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**Abstract**

Radiation resistance represents an imperative obstacle in the treatment of patients with colorectal cancer, which remains difficult to overcome. Here, we explored the anti-proliferative and migration-inhibiting properties of the natural product shikonin on a radiation-resistant human colon carcinoma cell line (SNU-CSRR). Shikonin reduced the viability of these cells in a dose-dependent manner; 38 μM of shikonin was determined as the half-maximal inhibitory concentration. Shikonin induced apoptotic cell death, as demonstrated by increased apoptotic body formation and the number of TUNEL-positive cells. Moreover, shikonin enhanced mitochondrial membrane depolarization and Bax expression and also decreased Bcl-2 expression with translocation of cytochrome c from mitochondria into the cytosol. In addition, shikonin activated mitogen-activated protein kinases, and their specific inhibitors reduced the cytotoxic effects of shikonin. Additionally, shikonin decreased the migration of SNU-CSRR cells via the upregulation of E-cadherin and downregulation of N-cadherin. Taken together, these results suggest that shikonin induces mitochondria-mediated apoptosis and attenuates epithelial-mesenchymal transition in SNU-CSRR cells.

**Key Words:** Shikonin, Radiation resistance, Human colon cancer, Epithelial to mesenchymal transition, Mitochondria-mediated apoptosis, Mitogen-activated protein kinases

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Cell viability was measured using the MTT assay. *4-methylpent-3-enyl)naphthalene-1,4-dione]. (B) SNU-C5 or SNU-C5RR cells were treated with 10, 20, 30, 40, or 50 μM shikonin for 24 h.

Materials and Methods

Reagents

Shikonin (5,8-dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl)naphthalene-1,4-dione, Fig. 1A) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33342, trypan blue solution, 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-phospho-JNK, anti-JNK, anti-phospho-p38, and anti-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Anti-phospho-JNK, anti-JNK, anti-phospho-p38, and anti-caspase-3 antibodies along with SB203580 (p38 inhibitor), and U0126 (ERK inhibitor) were obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). Anti-Bax, anti-Bcl-2, anti-caspase-9, anti-cytochrome c oxidase subunit 4 (COX4), anti-phospho-ERK, anti-ERK, anti-p38, anti-E-cadherin, and anti-N-cadherin antibodies were supplied by Santa Cruz Biotechnology, Inc (Dallas, TX, USA). The JNK inhibitor SP600125 was provided by Selleck Chemicals (Houston, TX, USA). All other chemicals and reagents used were of analytical grade.

Cell culture

Human colorectal adenocarcinoma (SNU-C5) and radiation-resistant (SNU-C5RR) colon cancer cell lines were incubated in a humidified 5% CO2 atmosphere at 37°C in RPMI medium containing 10% heat-inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/mL), and streptomycin (100 μg/mL).

Cell viability assay

SNU-C5 and SNU-C5RR cells were treated with various concentrations of shikonin and then incubated for 24 h. In addition, SNU-C5RR cells were treated with shikonin, SB203580, SP600125, U0126, or their combinations, and then incubated for 24 h. To evaluate the EMT in SNU-C5RR cells, cell viability was assessed after 48 h of shikonin treatment. After each treatment, exactly 125 μL of MTT solution (2 mg/mL) was added to each well. After maintaining the cells at 37°C until the formation of formazan crystals, the supernatant was removed by aspiration. Then, we dissolved the formed formazan crystals in dimethyl sulfoxide solution and read the relevant absorbance values at 540 nm with a scanning multi-well spectrophotometer (Shilnikova et al., 2018).

Colony formation assay

SNU-C5RR cells were cultured in 60 mm dishes, and 400 cells per dish were used as the optimum cell density. Cultured cells were incubated, and 38 μM shikonin was added on day 7 of incubation. The cell culture medium was changed every 3 days. On day 11 of incubation, 70% ethanol was applied to fix the cells, which were then stained with 0.4% trypan blue until colonies were detectable. Colony formation was imaged and quantified using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

Fig. 1. Shikonin induces cell death in SNU-C5 and SNU-C5RR cells. (A) The chemical structure of shikonin [5,8-dihydroxy-2-(1-hydroxy-4-methylpent-3-enyl)naphthalene-1,4-dione]. (B) SNU-C5 or SNU-C5RR cells were treated with 10, 20, 30, 40, or 50 μM shikonin for 24 h. Cell viability was measured using the MTT assay. **p<0.05 vs the control group in SNU-C5 and SNU-C5RR cells, respectively. (C) For the colony formation assay, SNU-C5RR cells were cultured for 11 days and treated with 38 μM shikonin on day 7. Colonies containing >400 cells were counted. *p<0.05 vs the control group.
Nuclear staining with Hoechst 33342
After treating SNU-C5RR cells with 38 μM shikonin or SP600125 along with SB203580, U0126, or a combination for 24 h, Hoechst 33342 staining was performed, followed by incubation at 37°C for 10 min. Then, cells were observed using a fluorescence microscope (Olympus Life Science, Tokyo, Japan).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay
SNU-C5RR cells (1×10^4 cells/mL) were cultured in chamber slides and maintained for 24 h, 38 μM Shikonin was added, and cells were incubated for 24 h. The slides were then prepared following the manufacturer’s instructions for the DeadEnd Colorimetric TUNEL system (Promega Corporation, Madison, WI, USA). Farnam mounted aqueous mounting medium (Agilent Technologies, Inc., Santa Clara, CA, USA) was used to mount stained cells on microscope slides. The microscopic images were captured via confocal fluorescent microscopy using an appropriate Laser Scanning Microscope 5 PASCAL program (Carl Zeiss AG, Oberkochen, Germany).

Single-cell gel electrophoresis (Comet assay)
Cell pellets were suspended in 1% low-melting agarose (LMA), and the cell-containing suspension was evenly spread on a microscopic slide, which was pre-coated with 1% normal melting agarose. Slides were kept at 4°C to enhance the solidification of agarose and covered with 170 μL of 0.5% LMA. Then, slides were dipped in lysis buffer (2.5 M NaCl, 100 mM Na₃EDTA, 1% Triton X-100, 10 mM Tris, and 1% N-lauroyl sarcosinate, pH 10) for 1 h in dark at 4°C. Each slide was subjected to gel electrophoresis, which contained NaOH (300 mM) and Na₃EDTA (10 mM, pH 10). Electrophoresis (300 mA, 37°C to enhance the staining for another 15 min. Microscopic images were captured in the dark for 30 min, resulting in DNA unwinding and the expression of alkali-labile damage. After electrophoresis, slides were immersed in neutralizing buffer (0.4 M Tris, pH 7.5) and 70% ethanol. Immediately before detection, slides were stained with 40 μL of ethidium bromide (10 μg/mL) and visualized under a fluorescence microscope (Olympus Life Science), and images were gathered using an image analyzer (Komet 5.5, Kinetic Imaging, Bromborough, UK). Exactly 25 cells per slide were used to quantify the comet tail length and total fluorescence percentage of the tails.

Mitochondrial membrane potential (Δψm)
SNU-C5RR cells were incubated and treated with 38 μM shikonin, then dyed with JC-1 (10 μg/mL) and maintained at 37°C to enhance the staining for another 15 min. Microscopic slides were prepared using a mounting medium (DAKO, Carpinteria, CA, USA). The mitochondrial polarization and depolarization status were detected via confocal microscopy, which was implemented with the Laser Scanning Microscope 5 PASCAL software. In addition, data obtained via fluorescent microscopy were confirmed using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting
SNU-C5RR cells were lysed using PRO-PREP™ protein extraction solution (Intron Biotechnology, Inc., Seongnam, Korea). Protein concentrations were quantified using a Bio-Rad protein assay reagent kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were subjected to electrophoresis and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Nitrocellulose membranes were blocked in 2% fetal bovine serum (shaking), and incubated with primary antibodies (dilution, 1:1,000) overnight at 4°C. The membranes were further incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat antimouse IgG antibodies (Pierce; Thermo Fisher Scientific, Inc.; dilution, 1:5,000) for 1 h at room temperature. Corresponding protein bands were detected using an increased chemiluminescence western blotting detection kit (GE Healthcare Life Sciences, Little Chalfont, UK) and then exposed to X-ray films.

Cell migration assay
SNU-C5RR cells (1×10^4 cells/mL) were seeded on 100 mm dishes, incubated for 24 h, and then treated with 3 μM shikonin. Then, scratch lines (diameter, 1.5 mm) were established across the base of the culture dishes. The line areas were measured after 0 and 48 h using a fluorescence microscope (Olympus Life Science). The index of gap width was calculated as follows: (mean gap width, mm)/mean gap width in control, mm).

Detection of superoxide anion
The xanthine/xanthine oxidase system was used to generate superoxide anions, which interacted with DMPO, and the produced DMPO-OH adducts were measured using an ESR spectrometer. The ESR spectrum was recorded 2.5 min after PBS (pH 7.4) was mixed with 0.02 mL of 3 M DMPO, 5 mM xanthine, 0.25 U xanthine oxidase, and 3 μM shikonin. ESR spectrometer parameters were as follows: Central magnetic field, 336.8 mT; power, 5.00 mW; frequency, 9.4380 GHz; modulation width, 0.2 mT; amplitude, 1000; sweep width, 0 mT; sweep time, 0.5 min; time constant, 0.03 s; temperature, 25°C.

Detection of hydroxyl radicals
Hydroxyl radicals were generated through the Fenton reaction (H₂O₂+FeSO₄) and allowed to interact with DMPO. The resultant DMPO-OH adducts were detected using an ESR spectrometer (Chae et al., 2011; Wu et al., 2012). The ESR spectrum was recorded 2.5 min after PBS (pH 7.4) was mixed with 0.02 mL of 0.3 M DMPO, 10 mM FeSO₄, 10 mM H₂O₂, and 3 μM shikonin. ESR spectrometer parameters were as follows: Central magnetic field, 336.8 mT; power, 1.00 mW; frequency, 9.4380 GHz; modulation width, 0.2 mT; amplitude, 600; sweep width, 10 mT; sweep time, 0.5 min; time constant, 0.03 s; temperature, 25°C.

Intracellular reactive oxygen species (ROS) level
SNU-C5RR cells were seeded on a coverslip-containing four-well plate at a density of 1.5×10^4 cells/well, and then cells were treated with 3 μM shikonin. After 24 h, representative cell groups were treated with DCF-DA (final concentration 20 μM) and incubated for 30 min at 37°C. Stained cells were washed with PBS, and the coverslips were mounted onto microscope slides in a mounting medium (DAKO). Then, the slides were analyzed using a confocal microscope. Relevant images were gathered using the Laser Scanning Microscope 5 PASCAL software (Carl Zeiss, Jena, Germany). In addition, data obtained via fluorescent microscopy were confirmed using a FACSCalibur flow cytometer (BD Biosciences).
Shikonin initiates mitochondria-mediated apoptosis

Mitochondria in control cells displayed red fluorescence upon staining with JC-1, which was visualized through $\Delta_{\psi m}$ polarization via confocal microscopy. However, shikonin-treated cells displayed a decrease in mitochondrial red fluorescence and elevated green fluorescence, signifying $\Delta_{\psi m}$ depolarization (Fig. 3A). The microscopic images were consistent with the flow cytometry analysis, confirming that shikonin destroyed $\Delta_{\psi m}$ (Fig. 3B). Furthermore, we investigated the expression of proteins associated with mitochondria-mediated apoptosis. Shikonin enhanced the expression of the pro-apoptotic protein Bax and suppressed the expression of pro-survival/anti-apoptotic protein Bcl-2 (Fig. 3C), leading to the translocation of cytochrome c from mitochondria into the cytosol (Fig. 3D). In addition, shikonin enhanced the expression of active (cleaved) caspase-9 and caspase-3 (Fig. 3E).

Shikonin decreased EMT progression in radiation-resistant colon carcinoma cells

Next, we explored the effect of shikonin on the migration of SNU-C5RR cells to understand its impact on EMT. First, we used a non-toxic concentration of shikonin, which allowed the observation of migrating cells up to 48 h after treatment. MTT data showed that $3 \mu M$ shikonin did not decrease the
viability of SNU-C5RR cells 48 h after treatment (Fig. 5A); therefore, we used this concentration in subsequent studies of cellular mobility. We observed wounds established at the base of culture dishes 0 and 48 h after treatment with 3 μM shikonin. While the gap was reduced in the control group, it was expanded in the shikonin-treated group (Fig. 5B). We then determined the expressions of E- and N-cadherin, glycoproteins involved in initiating and stabilizing intercellular contacts. Expression of the epithelial marker E-cadherin and interstitial marker N-cadherin was higher and lower, respectively, after shikonin treatment compared to that in untreated cells (Fig. 5C), confirming that shikonin attenuated EMT and thus the metastatic capacity of SNU-C5RR cells. Increased scavenging of superoxide anions and hydroxyl radicals by 3 μM shikonin was observed in the cell-free system (Fig. 5D, 5E). Furthermore, shikonin reduced cellular ROS production in SNU-C5RR cells, detected using confocal microscopy and flow cytometry after staining with DCF-DA (Fig. 5F, 5G).

**DISCUSSION**

Our investigation focused on radioresistance and ways to overcome it in colorectal cancer. We found that the herbal compound shikonin significantly suppressed the viability of radiation-resistant human colon carcinoma cells, and we attempted to identify an appropriate mechanism of apoptosis and EMT attenuation initiated by shikonin.

Apoptosis is a type of cell death that leads to several morphological and metabolic alterations, including chromatin condensation, nuclear fragmentation, and activation of special mediators (Wlodkowic et al., 2011). Thereby, the induction of...
apoptosis is an important strategy for anticancer therapy. Accumulating evidence shows that various apoptotic agents induce single- and double-strand DNA breaks (Lim et al., 2011). Our experiments indicated that the apoptotic body indices and the number of TUNEL-positive cells were elevated in shikonin-treated cells, as well as DNA single- and double-strand breaks, detected via comet assay. These results showed that the cytotoxic effect of shikonin is mediated through the apoptotic mechanism.

Numerous factors induce apoptosis via mitochondria-related pathways (Xiong et al., 2014). Oxidative stress or similar stress inducers destroy Δψm, resulting in the outflow of cytochrome c from mitochondria into the cytosol. These events are controlled by pro-survival/anti-apoptotic Bcl-2 and the pro-apoptotic protein Bax, leading to apoptosome complex formation. This complex initiates the caspase cascade and induces apoptosis (Yang et al., 2017). Thus, the typical markers of mitochondria-related apoptotic cell death include upregulation of Bax, downregulation of Bcl-2, mitochondrial membrane depolarization, the release of cytochrome c from mitochondria, apoptotic body formation, and activation of the caspase cascade (Wang et al., 2016). Here, we detected the loss of Δψm following shikonin treatment. Moreover, Bcl-2 expression was suppressed, whereas Bax expression increased. In addition, we observed the leakage of cytochrome c from mitochondria into the cytosol and the activation of caspase-9 and caspase-3. These data provide evidence of mitochondria-related apoptosis induced by shikonin.

Many recent investigations have demonstrated that the cytotoxic properties of most compounds with anticancer activities are recognized through the activation of MAPK family members (Sun et al., 2015). Here, we focused on the role of MAPKs in apoptotic cell death induced by shikonin. The expression of activated JNK, p38, and ERK was enhanced with time upon treatment with shikonin, whereas the specific MAPK inhibitors demonstrated a cytoprotective effect against shikonin-induced cell death. Thus, our findings confirm that shikonin induces apoptotic cell death in SNU-C5RR cells via the activation of MAPK cascades.

EMT is closely related to tumor metastasis (Todosi et al., 2016). EMT is closely related to tumor metastasis (Todosi et al., 2016).
2012; Zhu et al., 2013; Ameli and Khalily, 2016; Matějka et al., 2017). This process is marked by the downregulation of epithelial markers (E-cadherin and β-catenin) and upregulation of mesenchymal markers (N-cadherin and fibronectin). Changes in N-cadherin expression, as well as the EMT E-cadherin/N-cadherin switch, are known as independent risk factors for cancer growth and metastasis (Di Domenico et al., 2011). These findings have been fully explored in gastric, prostate, and oral carcinoma cell lines (Di Domenico et al., 2011; Feng et al., 2017; Ramamurthy et al., 2018), and there are many reports on EMT in colon cancer (Todosi et al., 2017; Ramamurthy et al., 2018), and there

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be considered a potential candidate to overcome radiation re-

activates apoptosis and attenuates EMT. Thus, shikonin can

lon cancer cells are sensitive to shikonin and that shikonin

significantly decreased ROS levels in SNU-C5RR cells, exerting

konin may be related to its antioxidant effect. Shikonin sig-

of Akt/Src/Erk pathways (Kim et al., 2016; Ahn et al., 2017). ROS induce EMT by activating Snail expression and inhibits E-cadherin expression in MCF-7 cells (Lee et al., 2019), as well as mediate EMT and cell invasive potential in colon cancer (Jiao et al., 2016; Kang et al., 2018). ROS also act as a key signaling molecule mediating the fractionated ionizing radiation-induced EMT response through the activation of Akt/Src/Erk pathways (Kim et al., 2020).

Thus, we suggest that the EMT-attenuating property of shikoni

significantly decreased ROS levels in SNU-C5RR cells, exerting anti-EMT effects, as increased ROS levels are important for EMT (Wang et al., 2010; Cichon and Radisky, 2014). Taken together, these results suggest that SNU-C5RR colon cancer cells are sensitive to shikonin and that shikonin activates apoptosis and attenuates EMT. Thus, shikonin can be considered a potential candidate to overcome radiation resistance in colon cancer.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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REFERENCES

Ahn, H. M., Yoo, J. W., Lee, S., Lee, H. J., Lee, H. S. and Lee, D. S. (2017) Peroxiredoxin 5 promotes the epithelial-mesenchymal transition in colon cancer. Biochem. Biophys. Res. Commun. 487, 580-586.

Ameli, M. and Khalily, F. (2016) Analysis of colorectal cancer and polyp for presence herpes simplex virus and cytomegalovirus DNA sequences by polymerase chain reaction. J. Anal. Res. Clin. Med. 4, 82-89.

Andújar, I., Rios, J. L., Giner, R. M. and Recio, M. C. (2013) Phar-

macological properties of shikonin - a review of literature since 2002. Planta Med. 79, 1685-1697.

Baskar, R., Lee, K. A., Yeo, R. and Yeoh, K. W. (2012) Cancer and radiotherapy: current advances and future directions. Int. J. Med. Sci. 9, 193.

Chae, S., Piao, M. J., Kang, K. A., Zhang, R., Kim, K. C., Youn, U. J., Nam, K. W., Lee, J. H. and Hyun, J. W. (2011) Inhibition of matrix metalloproteinase-1 induced by oxidative stress in human keratinocytes by mangiferin isolated from Anemarrhena asphodeloi-
des. Biosci. Biotechnol. Biochem. 75, 2321-2325.

Cichon, M. A. and Radisky, D. C. (2014) ROS-induced epithelial-mesenchymal transition in mammary epithelial cells is mediated by NF-κB-dependent activation of Snail. Oncotarget 5, 2827-2838.

Di Domenico, M., Pierantoni, G. M., Feola, A., Esposito, F., Laino, L., DE Rosa, A., Rullo, R., Mazzotta, M., Martano, M., Sanguedolce, F., Perillo, L., D’Angelo, L., Papagerakis, S., Tortorella, S., Bufo, P., Lo Muzio, L., Pannone, G. and Santoro, A. (2011) Prognostic significance of N-Cadherin expression in oral squamous cell carci-

noma. Anticancer Res. 31, 4211-4218.

Feng, L. M., Wang, X. F. and Huang, Q. X. (2017) Thymoquinone in-

duces cytotoxicity and reprogramming of EMT in gastric cancer cells by targeting PI3K/Akt/mTOR pathway. J. Biol. 42, 547-554.

Han, C. T., Kim, M. J., Moon, S. H., Jeon, Y. R., Hwang, J. S., Nam, C. C., Park, C. W., Lee, S. H., Na, J. B., Park, C. S., Park, H. W., Lee, J. M., Jang, H. S., Park, S. H., Han, K. G., Choi, Y. W., Lee, H. Y. and Kang, J. K. (2015) Acute and 28-day subacute toxicity studies of hexane extracts of the roots of Lithospermum erythrorhizon in Sprague-Dawley rats. Toxicol. Res. 31, 403-414.

He, G., He, G., R., Pi, Z., Zhu, T., Jiang, L. and Xie, Y. (2016) Enhancement of cisplatin-induced colon cancer cells apoptosis by shikonin, a natural inducer of ROS in vitro and in vivo. Biochem. Biophys. Res. Commun. 469, 1075-1082.

Jeung, Y. J., Kim, H. G., Ahn, J., Lee, H. J., Lee, S. B., Won, M., Jung, C. R., Im, J. Y., Kim, B. K., Park, S. K., Son, M. J. and Chung, K. S. (2016) Shikonin induces apoptosis of lung cancer cells via activation of FOXO3a/EGR1/SIRT1 signaling antagonized by p300. Biochim. Biophys. Acta 1863, 2584-2593.

Jiao, L., Li, D. D., Yang, C. L., Peng, R. Q., Guo, Y. Q., Zhang, X. S. and Zhu, X. F. (2016) Reactive oxygen species mediate oxaliplatin-induced epithelial-mesenchymal transition and invasive potential in colon cancer. Tumour Biol. 37, 8413-8423.

Jin, H., Gao, S., Guo, H., Ren, S., Ji, F., Liu, Z. and Chen, X. (2016) Re-sensitization of radiation resistant colorectal cancer cells to radi-

ination through inhibition of AMPK pathway. Oncol. Lett. 11, 3197-3201.

Kamiya, T., Goto, A., Kurokawa, E., Hara, H. and Adachi, T. (2016) Cross talk mechanism among EMT, ROS, and histone acetylation in phorbol ester-treated human breast cancer MCF-7 cells. Oxid. Med. Cell. Longev. 2016, 1284372.

Kang, K. A., Ryu, Y. S., Piao, M. J., Shilnikova, K., Kang, H. K., Yi, J. M., Boulander, M., Pacillo, R., Bossis, G., Yoon, S. Y., Kim, S. B. and Hyun, J. W. (2018) DUOX2-mediated production of reactive oxygen species induces epithelial mesenchymal transition in 5-fluorouracil resistant human colon cancer cells. Redox Biol. 17, 224-235.

Kihl, H., Kim, S. M., Hong, S., Gil, H. M., Cheon, E., Lee, D. H., Kim, Y. A. and Keum, N. (2021) Time trends of colorectal cancer incidence and associated lifestyle factors in South Korea. Sci. Rep. 11, 2413.

Kim, S., Kim, S. H. and Lee, C. E. (2020) SOCS1 represses fraction-

ated ionizing radiation-induced EMT signaling pathways through the counter-regulation of ROS-scavenging and ROS-generating systems. Cell. Physiol. Biochem. 54, 1026-1040.

Ko, H., Kim, S. J., Shim, S. H., Chang, H. and Ha, C. H. (2016) Shi-

konin induces apoptotic cell death via regulation of p53 and Nrf2 in AGS human stomach carcinoma cells. Biomol. Ther. (Seoul) 24, 501-509.

Lee, S. Y., Ju, M. K., Jeon, H. M., Lee, Y. J., Kim, C. H., Park, H. G., Han, S. I. and Kang, H. S. (2019) Reactive oxygen species induce epithelial-mesenchymal transition, glycolytic switch, and mi-

chondrial repression through the Dlk2/Snail signaling pathways in MCF-7 cells. Mol. Med. Rep. 20, 2339-2346.

Lim, S. W., Ting, K. N., Bradshaw, T. D., Zeehanath, N. A., Wiart, C.,
Acalypha wilkesiana extracts induce apoptosis by causing single and double strand DNA breaks. J. Ethnopharmacol. 138, 616-623.

β-Hydroxyisovaleryl-shikonin induces human cervical cancer cell apoptosis via PI3K/AKT/mTOR signaling. Oncol. Lett. 10, 3434-3442.

Epithelial-mesenchymal transition in tumor tissue and its role for metastatic spread of cancer. Klin. Onkol. 30, 20-27.

The retinamide VNLG-152 inhibits f-AR/AR-V7 and MNK-eIF4E signaling pathways to suppress EMT and castration-resistant prostate cancer xenograft growth. FEBS J. 285, 1051-1063.

Shikonin induces mitochondria-mediated apoptosis and attenuates epithelial-mesenchymal transition in cisplatin-resistant human ovarian cancer cells. Oncol. Lett. 15, 5417-5424.

Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J. Recept. Signal Transduct. Res. 35, 600-604.

Colon cancer at the molecular level—usefulness of epithelial-mesenchymal transition analysis. Rev. Med. Chr. Soc. Med. Nat. Iasi. 116, 1106-1111.

Anti-leukemic activity of shikonin: role of ERPS5 in shikonin induced apoptosis in acute myeloid leukemia. Cell. Physiol. Biochem. 39, 604-616.

The EMT spectrum and therapeutic opportunities. Mol. Oncol. 11, 878-891.

Regulation of EMT in colorectal cancer: a culprit in metastasis. Cancers (Basel) 9, 171.

The relationship between the Bcl-2/Bax proteins and the mitochondria-mediated apoptosis pathway in the differentiation of adipose-derived stromal cells into neurons. PLoS ONE 11, e0163327.

Apoptosis and beyond: cytometry in studies of programmed cell death. Methods Cell Biol. 103, 55-98.

Differences in incidence and mortality trends of colorectal cancer worldwide based on sex, age, and anatomic location. Clin. Gastroenterol. Hepatol. 19, 955-966.

Mitochondria-mediated apoptosis in mammals. Protein Cell 5, 737-749.

Natural pyrethrins induces apoptosis in human hepatocyte cells via Bax-and Bcl-2-mediated mitochondrial pathway. Chem. Biol. Interact. 262, 38-45.

Epithelial-mesenchymal transition and its role in the pathogenesis of colorectal cancer. Asian Pac. J. Cancer Prev. 14, 2689-2698.

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