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Original Article

Effect of *Lycii cortex radicis* Extraction on Glioma Cell Viability

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Objectives: Little information is available regarding the effect of *Lycii cortex radicis* (LCR) on cell viability in glioma cells. This study was therefore undertaken to examine the effect of LCR on cell survival in U87MG human glioma cells.
Methods: Cell viability and cell death were estimated by MTT assay and trypan blue exclusion assay, respectively. Reactive oxygen species (ROS) generation was measured using the fluorescence probe DCFH-DA. Activation of Akt and extracellular signal-regulated kinase (ERK) and activation of caspase-3 were estimated by Western blot analysis.
Results: LCR resulted in apoptotic cell death in a dose- and time-dependent manner. LCR increased reactive oxygen species (ROS) generation and LCR-induced cell death was also prevented by antioxidants, suggesting that ROS generation played a critical role in LCR-induced cell death. Western blot analysis showed that LCR treatment caused down-regulation of Akt and ERK. The LCR-induced cell death was increased by the inhibitors of Akt and ERK. Activation of caspase-3 was stimulated by LCR and caspase inhibitors prevented the LCR-induced cell death.
Conclusion: These findings suggest that LCR results in human glioma cell death through a mechanism involving ROS generation, down-regulation of Akt and ERK, and caspase activation.

Key Words : *Lycii cortex radicis*, cell viability, ROS, Akt, ERK, human glioma cells.

Introduction

Glioblastoma is the most common and highly aggressive primary brain tumor and is characterized by marked angiogenesis and extensive tumor cell invasion into the normal brain parenchyma[1,2]. Glioblastoma is a rapidly growing, highly infiltrative tumor making complete surgical removal impossible. After diagnosis of glioblastoma multiforme, the median survival time of 9-12 months has remained unchanged; despite aggressive treatments including surgical removal of the tumor, radiotherapy, and chemotherapy, this statistic has not changed significantly over the past years[3,4]. Since the location of tumors in the brain limits drug availability, the use of novel therapeutic approaches may be required.

Natural products derived from plants have recently received much attention as potential chemopreventive and chemotherapeutic agents. Among them, great attention has been given to natural products with polyphenols. These substances appear very promising for cancer prevention and treatment in preclinical models and clinical trials[5-7]. The use of synthetic agents in long-term chemopreventive strategies is associated with toxicity problems, and development of multidrug resistance further limits chemotherapy's

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effectiveness. Therefore, oriental medicinal drugs may be a very promising group of compounds, exerting chemopreventive and chemotherapeutic effects.

Lycii cortex radicis, (LCR) has been extensively used in traditional Korean medicine for centuries due to its biological activities, such as cooling blood and bringing down fever[8]. Previous studies have shown that LCR extracts improve insulin resistance and lipid metabolism in obese-diabetic rats[9]. However, little information is available regarding the effect of LCR on glioma cell viability.

This study was undertaken to investigate if LCR affected cell viability and to characterize its molecular mechanism in U87MG human glioma cells. The results show that LCR induced apoptosis through down-regulation of ERK and Akt pathway.

These data suggest that LCR may be a potential candidate for both glioblastoma prevention and treatment.

Materials and Methods

1. Reagents

N-acetylcysteine (NAC), Trolox, Hoecst33258, 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT), and propidium iodide were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). Tween 20, U0126, LY984002, VAD-FMK, and DEVD-CHO were purchased from Calbiochem (San Diego, CA, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR, USA). Antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). All other chemicals were of the highest commercial grade available.

2. Preparation of LCR extractions

The crushed *Lycii cortex radicis* (1,000 g) was extracted 3 times, each time with 3 volumes of methyl alcohol, at 60℃ for 24h. The extract was filtered and evaporated under reduced pressure using a rotary evaporator to yield 72.97 g (yield 7.30%).

3. Cell culture

U87MG cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in $75cm_2$ culture flasks (Costar, Cambridge, MA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Invitrogen, Carlsbad, CA, USA) containing 10% heat inactivated fetal bovine serum (HyClone, Logan, UT, USA) at 37oC in humidified 95% air / 5% CO_2 incubator. When the cultures reached confluence, a subculture was prepared using a 0.02% EDTA-0.05% trypsin solution. The cells were grown on well tissue culture plates and used 1-2 days after plating when a confluent monolayer culture was achieved. Unless otherwise stated, cells were treated with LCR in a serum-free medium. Test reagents were added to the medium 30 min before LCR exposure.

4. Measurement of cell viability and cell death

Cell viability was evaluated using an MTT assay[10]. After washing the cells, culture medium containing 0.5 mg/ml of MTT was added to each well. The cells were incubated for 2 hrs at 37oC, the supernatant was removed and the formazan crystals formed in viable cells were solubilized with 0.11 ml of dimethyl sulfoxide. A 0.1 ml aliquot of each sample was then translated to 96-well plates and the absorbance of each well was measured at 550 nm with ELISA Reader (FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany). Data were expressed as a percentage of control measured in the absence of LCR. Unless otherwise stated, the cells were exposed to 50 μM LCR for 48 hrs. Test reagents were added to the medium 30 min before LCR exposure.

Cell death was estimated by counting the cell number and trypan blue exclusion assay, respectively. The cells were harvested using 0.025% trypsin and

incubated with 4% trypan blue solution. The number of viable and nonviable cells was counted using a hemocytometer under light microscopy. Cells failing to exclude the dye were considered nonviable.

5. Measurement of apoptosis

1) Cytochemical staining: Cells were grown in 6-well plates; after treatment with stimuli, the cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 hr at 4℃. Then the cells were stained with 10 mM Hoechst 33258 for 15 min at 37℃. Cells were then washed twice with PBS and examined by confocal microscopy (LSM510, ZEISS, Germany).

2) Cell cycle analysis: Cells were grown in 6-well plates and were treated as indicated. Then, attached and floating cells were pooled, pelleted by centrifugation, washed in PBS, and fixed with cold 70% ethanol containing 0.5% Tween 20 at 4℃ overnight. Cells were washed and resuspended in 1.0 ml of propidium iodide solution containing 100 mg of RNase A/ml and 50 mg propidium iodide/ml and incubated for 30 min at 37℃. Apoptotic cells were assayed using FACSort Becton Dickinson Flow Cytometer at 488 nm and the data were analyzed with CELLQuest Software. Cells with sub-G1 propidium iodide incorporation were considered as apoptotic. The percentage of apoptotic cells was calculated as the ratio of events on sub-G1 to events from the whole population.

6. Measurement of reactive oxygen species (ROS)

The intracellular generation of ROS was measured using DCFH-DA. The nonfluorescent ester penetrated into the cells and was hydrolyzed to DCFH by the cellular esterases. The probe (DCFH) was rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCF) in the presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Cells cultured in 24-well plate were preincubated in the culture medium with 30 mM DCFH-DA for 1 hr at 37oC. After the preincubation, the cells were exposed to 50 μM LCR for various durations. Changes in DCF fluorescence were assayed using FACSort Becton Dickinson Flow Cytometer (Becton-Dickinson Bioscience, San Jose, CA, USA) and the data were analyzed with CELLQuest Software.

7. Western blot analysis

Cells were harvested at various times after LCR treatment and disrupted in lysis buffer (1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4). Cell debris was removed by centrifugation at 10,000 g for 10 min at 4oC. The resulting supernatants were resolved on a 12% SDS-PAGE under denatured reducing conditions and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk at room temperature for 30 min and incubated with primary antibodies. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody. The signal was visualized using an enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

8. Measurement of caspase-3 activity

Caspase-3 activity was measured by Western blot analysis using the procaspase-3 specific antibody as described above.

9. Statistical analysis

The data was expressed as means ± SEM and the difference between two groups was evaluated using Student's t-test. Multiple group comparison was done using one-way analysis of variance followed by the Tukey post hoc test. A probability level of 0.05 was used to establish significance.

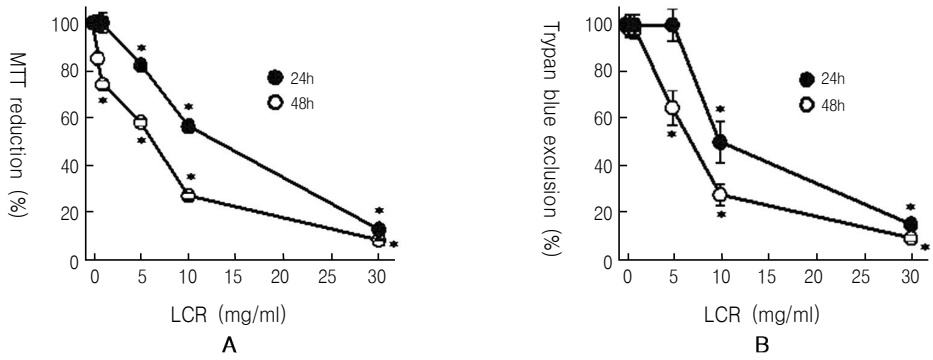

Fig. 1. Effect of *Lycii Cortex Radicis* (LCR) on cell viability and cell death.

Cells were exposed to various concentrations of LCR for 24 and 48 hrs (A). Cell viability was estimated by MTT reduction assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without LCR. (B) Cells were exposed to various concentrations of LCR for 24 and 48 hrs. Cell death was estimated by trypan blue exclusion assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with control without LCR.

Results

1. Effect of LCR on cell viability and cell death

To determine the effect of LCR on cell viability, cells were exposed to 1-30 mg/ml for 24 and 48 hrs. LCR caused loss of cell viability in a dose- and time-dependent manner as evidenced by a decrease in MTT reduction (Fig. 1A). After adding 10 mg/ml LCR for 24 and 48 hrs, the cell viability decreased approximately 55 and 25% of control, respectively. To ascertain whether the reduction in cell viability was attributed to the cell death, trypan blue exclusion assay was performed. As shown in Fig. 1B, changes in cell death by LCR were similar to those estimated by MTT assay, suggesting that the reduction in cell viability by LCR was mainly due to induction of cell death.

To determine whether the LCR-induced cell death resulted from apoptosis, cells were exposed to 10 mg /ml LCR for 24 hrs. Changes in nuclear morphology were evaluated using a specific DNA-binding fluorochrome (Hoechst 33258). As shown in Fig. 2A, the nuclei of normal (control) cells were big and round,

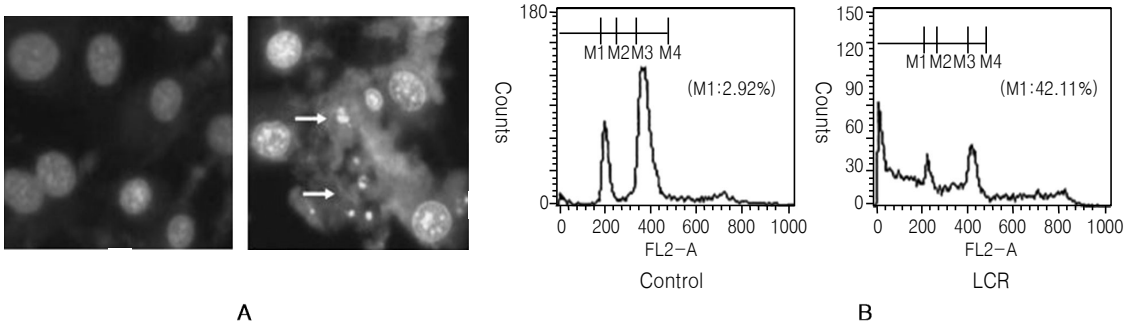

Fig. 2. Effect of *Lycii Cortex Radicis* (LCR) on apoptosis.

Cells were exposed to 10 mg/ml LCR for 24 hrs. Apoptosis was estimated by Hoechst33258 staining (A) and flow cytometric analysis (B). Arrows in (A) indicated cells with DNA fragmentation. In flow cytometric analysis, numbers indicated the percentage of cells with the sub-G1 peak (M1gated, apoptotic cells).

without condensation nor fragmentation, whereas the cells treated with LCR exhibited nuclear morphology with condensation and DNA fragmentation, the typical morphological features of apoptosis. To further verify the apoptosis, flow cytometric analysis was performed for cells exposed to LCR. The sub-G1 peak (M1 gate) that represents a population of cells with reduced DNA stainability, probably due to DNA fragmentation, increased from 2.92% in the control to 41.11% in the LCR-treated cells (Fig. 2B). These data suggest that the LCR-induce cell death was largely attributed to the induction of apoptosis.

2. Role of ROS in LCR-induced cell death

To determine whether LCR induces ROS generation in human glioma cells, the cells were exposed to LCR and changes in DCF fluorescence were measured by flow cytometry. ROS generation was increased in cells exposed to 10 mg/ml LCR for 12 hrs as assessed by increased DCFH-DA oxidation (Fig. 3A). To define whether ROS generation was involved in the LCR-induced cell death, the effect of antioxidants NAC and Trolox on the cell viability was examined. The LCR-induced loss of cell viability was prevented by these antioxidants (Fig. 3B), indicating that LCR-induced cell death was associated with ROS generation in human glioma cells.

3. Effect of LCR on activation of cell survival kinases

Akt and ERK play a pivotal role in cell proliferation, differentiation, and survival[11-13]. If LCR causes down-regulation of these kinases, cell death could be induced. To assess this possibility, activity of these kinases was evaluated by detecting their phosphorylation forms. Cells were exposed to 10 mg/ml LCR for various times and changes in activation of Akt and ERK were evaluated by Western blot analysis using antibodies specific to the respective phosphorylated form. As shown in Fig. 4A, LCR caused a decrease in activation of these kinases after 1 hr of treatment (Fig. 4A and B).

To evaluate whether down-regulation of these kinases was responsible for the LCR-induced cell death, the effect of inhibitors was evaluated. As shown in Fig. 4C, the Akt inhibitor LY984002 and the ERK inhibitor U0126 increased the cell death induced by LCR. These results suggest that down-regulation of Akt and ERK is responsible for the LCR-induced cell death.

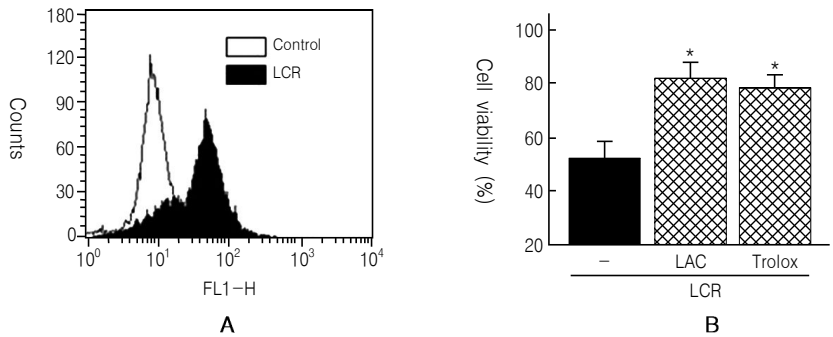

Fig. 3. Role of reactive oxygen species (ROS) generation in *Lycii Cortex Radicis* (LCR)-induced cell death.

(A) Cells were exposed to 10 mg/ml LCR for 12 hrs and the DCF fluorescence intensity was measured by a flow cytometer. (B) Effect of antioxidants on LCR-induced cell death. Cells were exposed to 10mg/ml LCR for 24 hrs in the presence or absence of 2mM N-acetylcysteine (NAC) and 0.5mM Trolox. Cell viability was estimated by MTT reduction assay. Data are mean ± SEM of four independent experiments performed in duplicate. *$p<0.05$ compared with LCR alone.

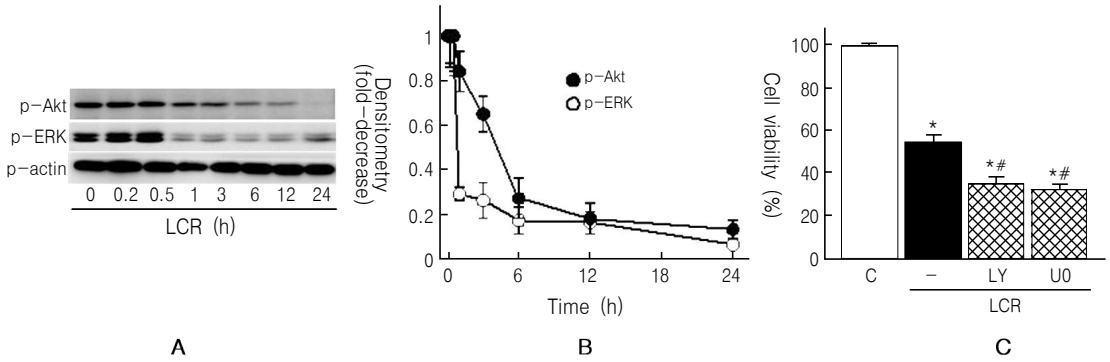

Fig. 4. Effect of Lycii Cortex Radicis (LCR) on activation of Akt and ERK.

Representative (A) and quantitative (B) were the results of three independent experiments. Cells were exposed to 10 mg/ml LCR for various times. β-Actin was employed as a loading control. (C) The effect of antioxidant on LCR-induced cell death. Cells were exposed to 10 mg/ml LCR for 24 hrs in the presence or absence of each 10 mM LY984002 (LY) and U0126 (U0). Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with LCR alone.

4. Role of caspase in LCR-induced cell death

Caspases play a key role during the execution phase in apoptosis and the caspase-3 is one of the executioners of apoptosis[14]. To determine if caspase activation was involved in the LCR-induced cell death, activity of caspase-3 was measured using Western blot analysis in cells exposed to 10 mg/ml LCR for various times. LCR decreased expression of pro-caspase-3 after 1 hr of treatment (Fig. 5A), suggesting that LCR caused the activation of caspase-3. To evaluate if caspase was involved in the LCR-induced cell death, the effect of caspase inhibitors on the cell viability was examined. As shown in Fig. 5B, the LCR-induced cell death was prevented by the caspase-3 inhibitor DEVD-CHO and the general caspase inhibitor VAD-FMK. These data indicate that LCR induced cell death through a caspase-dependent mechanism in human glioma cells.

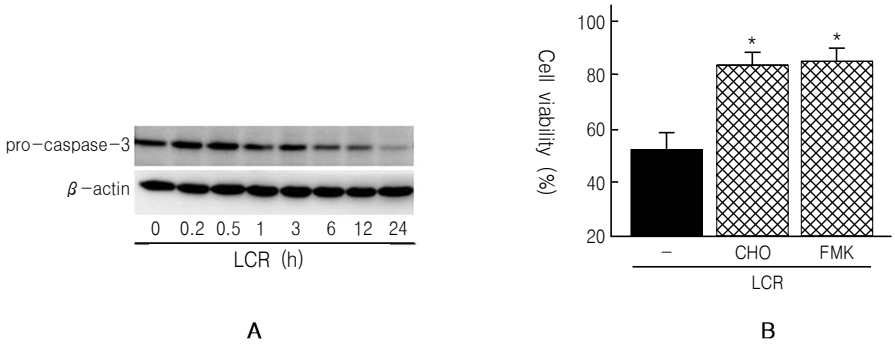

Fig. 5. Role of caspase activation on Lycii Cortex Radicis (LCR)-induced cell death.

Cells were exposed to 10 mg/ml LCR for various times (A) and caspase-3 activation was estimated by Western blot analysis. (B) The effects of caspase inhibitors on LCR-induced cell death. Cells were exposed to 10 mg/ml LCR for 24 hrs in the presence or absence of each 20 μM of DEVD-CHO (CHO) and VAD-FMK (FMK). Cell viability was estimated by MTT assay. Data are mean ± SEM of four independent experiments performed in duplicate. *p<0.05 compared with LCR alone.

Discussion

Inhibition of cancer cell growth through the induction of differentiation and apoptosis is an attractive approach to human cancer therapy. Previous studies have shown that natural products derived from plants exert anticancer effects via the induction of apoptosis, although the molecular mechanisms by which they induce apoptosis have not been yet clarified[15,16].

LCR have been reported to induce reduction in blood glucose levels[17], to prevent anaphylactic shock[18] and hyperlipidemia[19], and to have antioxidative action[20]. However, the effect of LCR on human glioma cell viability was not yet explored.

The present study demonstrated that LCR caused loss of cell viability in a dose-and time-dependent manner and its effect was attributed to cell death (Fig. 1). Morphological findings and flow cytometric analysis showed that LCR-induced cell also that the death was largely due to apoptosis (Fig. 2). These data indicated that LCR caused the loss of cell viability through apoptotic cell death in human glioma cells.

Flavonoids, the major component of natural products derived from plants, behave as an antioxidant[16] or a pro-oxidant generating ROS[21-23]. ROS generation by flavonoids is responsible for cell death in some cancer cells[23,24]. In the present study, LCR increased ROS generation (Fig. 3A). The LCR-induced cell death was prevented by antioxidants, NAC and Trolox, indicating that ROS production plays an important role in the LCR-induced cell death.

Since several lines of evidence described an important role for the activation of Akt and ERK in glial survival signaling and glial tumorigenesis[25-32], we examined the role of these kinases in the LCR-induced glioma cell death. The Akt signaling pathway mediated glioma cell survival and growth[29]. The aberrant activation of Akt signaling was identified as crucial to the malignant features of glioblastoma multiforme such as rapid tumor growth, invasiveness, resistance to cytotoxic treatments, and massive neovascularization[29-31]. Therefore, the regulation of the Akt signaling pathway would be a promising target for the clinical management of patients with glioma. In this regard, the modulation of Akt signaling may also be associated with LCR-induced cell death. Indeed, Akt was highly activated in U87MG human glioma cells and this Akt activation was inhibited by LCR (Fig. 4A and B). To clarify the role of Akt in cell death, the effect of LCR on cell viability was examined in the presence of the Akt inhibitor. The LCR-induced cell death was increased by the Akt inhibitor (Fig. 4C). These data imply that down-regulation of Akt played an important role in the LCR-induced glioma cell death. ERK is activated by a variety of extracellular signals including mitogens and contributes to the proliferative responses in cells, and is considered to be an essential common element of mitogenic signaling[13,32]. Its constitutive expression causes cell transformation and plays a putative role in the carcinogenesis process and drug resistance[33-35]. However, the role of ERK activation in the LCR-induced cell death was not clear. Activation of ERK was inhibited by flavonoids in vascular smooth muscle cells[36], human epidermal carcinoma cells[37], and neuronal cells[38], whereas it was increased following flavonoid treatment in lung cancer cells[39]. These studies suggested that the effect of flavonoids on ERK activation was dependent on cell types. In the present study, LCR caused an inhibition of ERK phosphorylation (Fig. 4A and B). The LCR-induced cell death was further increased by addition of the ERK inhibitor U0126 (Fig. 4C). These data implied that down-regulation of Akt and ERK signaling pathway played an important role in the LCR-induced glioma cell death.

Although little is known regarding the precise mechanism by which natural products such as flavonoids induce apoptosis, the activation of caspases is a central effector mechanism in various cancer cell

types[15].

In the present study, we explored if LCR causes cell death through a caspase-dependent mechanism. LCR induced the activation of caspase-3, and this LCR-induced cell death was prevented by caspase inhibitors (Fig. 5).

In conclusion, the present study demonstrates that LCR results in human glioma cell death through the down-regulation of Akt and ERK. Our results suggest that LCR could be considered a potential candidate for both glioblastoma prevention and treatment.

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