Compositional Analysis of Walnut Lipid Extracts and Properties as an Anti-cancer Stem Cell Regulator via Suppression of the Self-renewal Capacity

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Abstract Colon cancer is a leading cause of cancer-related deaths worldwide. Effects of walnut (*Juglans regia* L.) lipid extracts (WLEs) on the self-renewal capacity of cancer stem cells (CSCs) in colon cancer were investigated. The dominant component of WLEs was α -linoleic acid (64.6%), followed by α -linolenic acid (14.6%), and oleic acid (12.6%). A higher concentration of γ -tocopherol (37.1%) was also present than of α -tocopherol (0.6%). CD133⁺CD44⁺CSCs treated with WLEs showed inhibition of colony formation and sphere formation, indicating a decrease in the self-renewal capacity. Treatment with WLEs also resulted in down-regulation of protein levels, including Notch1, phospho-GSK3 β (p-GSK3 β), and β -catenin, which are associated with CSCs and the self-renewing capacity. WLEs rich in essential fatty acids and γ -tocopherol can exert therapeutic actions on colon cancer via targeting of CSCs.

Keywords: walnut lipid extract, colon cancer, cancer stem cell, self-renewal capacity

Introduction

Colon cancer is a major public health problem and the second leading cause of cancer deaths worldwide (1). Despite advances in understanding of tumor biology and improvements in chemotherapy efficacy, nearly half of colon cancer patients still experience tumor recurrence due to resistance to chemotherapy. Therefore, the critical role of cancer stem cells (CSCs) in progression and metastasis of colon cancer has been recognized. CSCs represent a small subset of rare cancer cells that have potential to initiate and propagate cancer cells, and to cause relapse of cancer. (2). CSCs possess characteristics, including self-renewal, cell differentiation, and tumorigenicity (3). Thus, identification of CSCs and elimination of the self-renewal capacity could represent an effective strategy for treatment of colon cancer.

To identify colon CSCs, the expression of different cell surface markers, including CD133, CD44, aldehyde dehydrogenase 1 (ALDH1), CD29, and CD 24 were used. In particular, the cell surface proteins CD133 and CD44 are considered key stem cell markers for identification of colon cancer initiating cells (4). CD133 (prominin-1) is a glycoprotein expressed on the cell surface and serves as a receptor for a currently unknown ligand. CD133⁺ normal stem cells have been found in a stem cell niche of the adult intestine, and

CD133⁺ has also been identified in neoplastic cells (5).

CD44 is a hyaluronan receptor and a multifunctional cell surface molecule that is involved in cell-cell interactions, and also plays a pivotal role in colonization of adult stem cells (6). Previously, a small CD133⁺ or CD44⁺ subset of CSCs was found to be capable of forming clonospheres in a serum-free sphere medium, and was able to initiate tumor growth in xenograft mouse models (2,7). Cells that expressed both CD133⁺ and CD44⁺ exhibited CSC characteristics and greater tumorigenicity than cells that expressed only CD133⁺ or CD44⁺.

Other receptors and cell signaling proteins have also been associated with CSCs. For example, Notch homolog 1 (Notch1) is a transmembrane receptor that plays a fundamental role in regulation of cell proliferation and apoptosis in many organs (8), and Notch proteins have been identified in stem/progenitor cell populations. Furthermore, Notch-activated genes have been shown to drive tumor growth mediated by expanding CSCs (9). The Wnt/ β -catenin signaling pathway has also been shown to play a key role in self-renewing CSCs (10) with levels of intracellular β -catenin modulated by glycogen synthase kinase3 β (GSK3 β) via ubiquitination and proteasomal degradation (11).

Walnuts (*Juglans regia* L.) are rich in essential fatty acids, vitamin E, flavonoids, and phenolic acids (12). Correspondingly, walnuts are

known to provide health benefits for many chronic diseases, including cardiovascular disease (13), and for brain health (14). In addition, walnuts have been shown to delay growth of breast, prostate, and colon cancers (15–17). However, it is unknown whether walnut lipid extracts (WLEs) affect the self-renewal capacity of colon CSCs. Therefore, the purpose of this study was to characterize WLEs and to investigate their effects on the self-renewal capacity of CD133⁺CD44⁺ CSCs.

Materials and Methods

Preparation of WLEs English walnuts (*J. regia*) were provided by the California Walnut Commission, Folsom, CA, USA in January of 2014. Whole walnuts (3 g) were ground with mortar and pestle to get fine powder and dissolved in 9 mL of chloroform and methanol (2:1, v/v). The mixture was incubated in a dry oven at 60°C for 15 min, then vortexed for 1 h. The mixture was cool down in the refrigerator overnight at 4°C until all the samples sit to the bottom of the tube. The supernatant was centrifuged (Hanil Science Industrial Co., Incheon, Korea) for 15 min at 700×g. The supernatant was transferred to a new tube while the remaining mixture was washed twice with 3 mL of chloroform and methanol (2:1, v/v) by centrifugation. After the mixture was centrifuged for 15 min at 700×g, the resulting supernatant was purified for 1 h with nitrogen purging for removal of methanol. The supernatant was then vortexed with 6 mL of chloroform and methanol (2:1, v/v) and 3.6 mL of 0.05 % H₂SO₄. After a final centrifugation step at 700×g for 15 min, the resulting supernatant was purged with nitrogen for 2 h. Purified WLEs were then transferred to tubes with nitrogen gas and stored at –80°C.

GC analysis of fatty acids with a flame ionization detector (FID)

An internal standard of triundecanoin (C11:0) was dissolved in isooctane and added to WLEs at a concentration of 1,000 ppm (w/v). After removal of the solvent using a nitrogen gas flow, fatty acids were derivatized to fatty acid methyl esters (FAME) using 14% borontrifluoride (BF₃)/MeOH. A Hewlett-Packard 7890A GC (Agilent Technology, Palo Alto, CA, USA) equipped with a FID and a SPTM-2560 capillary GC column (100 m×0.25 mm ID, 0.20 µm film) (Supelco, Inc., Folsom, CA, USA) was used. The oven temperature was started at 100°C for 4 min, then increased to 225°C at 3°C/min, and maintained at 225°C for 40 min. Temperatures of the injector and the detector were 225 and 260°C, respectively. The flow rate of the helium carrier gas was 0.8 mL/min, the injection volume was 1 µL, and the split ratio was 1:200. GC chromatogram peaks obtained for WLEs were identified based on comparison with retention times of a mixture of standard FAME (Sigma-Aldrich Co., St. Louis, MO, USA). Concentrations of each peak were quantified using an equivalent concentration of the internal standard.

HPLC analysis of tocopherols WLEs (0.1 g) were mixed with *n*-

hexane, then filtered using a PTFE membrane filter. HPLC was performed using a Jasco Pu-2089 Plus instrument (Hachioji, Tokyo, Japan) equipped with a fluorescence detector. Separation was performed using a µ-PorasilTM column (3.9×300 mm², 10 µm inner diameter) (Waters, Milford, MA, USA). The isocratic mobile phase was composed of hexane/isopropanol (99.8:0.2, v/v) applied at a flow rate of 2 mL/min. The oven temperature was 35°C. The detection wavelengths were 290 nm for excitation and 330 nm for emission, respectively. Standard calibration curves were generated for quantitative analysis of tocopherols present.

Cell lines and cell culture The human colon cancer cell line HCT116 was purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). HCT116 cells were cultured in McCoy's 5A medium (Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA) and 1% penicillin-streptomycin (100 U/mL and 100 mg/mL, respectively) (Invitrogen, Carlsbad, CA, USA). HCT116 cells were maintained in a humidified incubator at 37°C with a 5% CO₂.

Isolation of CD133⁺CD44⁺ HCT116 cells using Fluorescence-Activated Cell Sorting (FACS)

For characterization of colon CSCs, expression of the CSC markers, including CD133 and CD44, was detected using flow cytometry. Briefly, HCT116 cells were harvested, washed twice with phosphate-buffered saline (PBS), then incubated with Alexa Fluor 488-conjugated CD44 antibody (Cell Signaling, Danvers, MA, USA) and phycoerythrin (PE)-conjugated CD133 antibody (Miltenyi Biotec, Bergisch Gladbach, Germany) in the dark at 4°C. After 30 min, cells were washed with a FACS assay buffer prior to analysis using a FACS DiVa system (BD Bioscience, San Jose, CA, USA). As a positive control, the 20% of cells with the highest fluorescence levels was used. As a negative control, the 20% of cells with the lowest fluorescence levels was used.

Clonogenic assay Clonogenic assays were performed for analysis of the ability of single cells to form colonies as an indicator of reproductive integrity representing the self-renewal capacity of CSCs (18). Briefly, CD133⁺CD44⁺HCT116 cells were seeded in 6 well plates at 250 cells/well and treated with WLEs at 0.1, 0.5, and 1.0 mg/mL. After 10 days, colonies present were fixed using 0.9% NaCl and were stained with crystal violet (Sigma-Aldrich). Colonies containing more than 50 cells were counted and the plating efficiency (PE) for each well was calculated as: (number of colonies/total number of cells seeded per well)×100 (%) (18).

Sphere formation assay Sphere formation assays were performed as described previously (3) for characterization of the sphere-forming capacity of CSCs in a serum-free CSCs medium as an indicator of self-renewal. Briefly, 6 well plates were coated with poly-2(2-hydroxyethyl methacrylate) (poly HEMA) (Sigma-Aldrich) before CD133⁺CD44⁺ HCT116 cells (1×10⁴ cells/6 well plate) were plated and grown in a

Fig. 1. GC-FID profiles of fatty acids and HPLC characterization of tocopherols in walnut lipid extracts (WLEs). (A) WLEs were prepared from whole walnuts. The fatty acids palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), and eicosanoic acid (C20:1) were detected using GC-FID. (B) WLEs were prepared from whole walnuts. Levels of α -tocopherol and γ -tocopherol were detected using HPLC.

sphere medium containing 1:1 DMEM/F-12 medium (Welgene), 2% B27 (Invitrogen), 20 ng/mL human epidermal growth factor (EGF) (Peprotech, London, UK), and 40 ng/mL basic fibroblast growth factor (bFGF) (Peprotech). After 6 day, the number of spheres aggregated with more than 50 cells was counted under an inverted microscope (Nikon, Tokyo, Japan).

Western blot assay Western blot assays were performed as described previously (3). Briefly, HCT116 cells were lysed with RIPA buffer, then lysates were separated using SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked with a 5% non-fat dried milk solution or with a 5% bovine serum albumin (BSA) solution for 1 h. Membranes were subsequently incubated at 4°C overnight with β -catenin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p-GSK3 β (Cell Signaling), or Notch1 (Novus Biologicals, Littleton, CO, USA) antibodies. Detection of α -tubulin (Sigma-Aldrich) served as a loading control.

Statistical analysis All data were analyzed using GraphPad Prism software (GraphPad Inc., San Diego, CA, USA). Results were expressed as a mean $\pm$ standard error of the mean (SEM). Data were analyzed

using a one way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post hoc test for multiple comparisons. More than 3 independent experiments were performed. Differences were considered statistically significant at $p < 0.05$.

Results and Discussion

GC-FID analysis of fatty acids in WLEs Major fatty acids identified in WLEs using GC-FID are shown in a chromatogram in Fig. 1A. The extraction yield of WLEs from walnuts was 45%. Fatty acid compositions and saturation of fatty acids in WLEs are shown in Table 1. Major fatty acids were α -linoleic acid (C18:2, 64.6%), α -linolenic acid (C18:3, 14.6%), oleic acid (C18:1, 12.6%), palmitic acid (C16:0, 5.8%), and stearic acid (C18:0, 2.2%). Eicosanoic acid (C20:1) was also detected at 0.1% in WLEs, which also contained saturated fatty acids (SFAs, 8.0%), mono-unsaturated fatty acids (MUFAs, 12.8%), and poly-unsaturated fatty acids (PUFAs, 79.2%).

Although methods previously used for extraction and analysis of walnuts (*Juglans regia* L.) differed, reported compositions of walnut lipids were similar (19,20). Pereira *et al.* (20) reported that linoleic acid was the most abundant fatty acid detected in 6 *J regia* L.

Table 1. Fatty acid composition and saturation of WLEs

Fatty acid	Composition (%)
Palmitic acid (C16:0)	5.8±0.08 ¹⁾
Stearic acid (C18:0)	2.2±0.03
Oleic acid (C18:1)	12.6±0.04
α-linoleic acid (C18:2)	64.6±0.09
α-linolenic acid (C18:3)	14.6±0.07
Eicosaenoic acid (C20:1)	0.1±0.003
SFAs ²⁾	8.1±0.1
MUFAs ²⁾	12.8±0.1
PUFAs ²⁾	79.2±0.1

¹⁾Mean±standard error of the mean (n=3)
²⁾SFA, Saturated fatty acid; MUFA, Mono-unsaturated fatty acid; PUFA, Poly-unsaturated fatty acid

cultivars (Francquette, Lara, Marbot, Mayette, Mellanaise, and Parisienne), and oleic acid was the second most abundant. Concentrations of linoleic acid ranged from 55.51 to 60.30% and concentrations of oleic acid from 14.92 to 20.22%. Concentrations of both in this study were 64.6 and 12.6%, respectively, slightly lower and slightly higher concentrations, respectively, than in previous reports (19). Concentrations of other fatty acids detected in this study, including linolenic, palmitic, and stearic acids, were also similar to concentrations reported in other studies (19,20). In addition, proportions of SFAs (8.0%), MUFAs (12.8%), and PUFAs (79.2%) were comparable to ranges previously reported for these fatty acid groups (8-11%, 8-11%, and 69-76%, respectively) (19). Thus, methods used for the extraction and analysis of WLEs in this study were reliable.

HPLC analysis of WLEs tocopherols Tocopherols present in prepared WLEs were analyzed using HPLC and the resulting chromatogram is shown in Fig. 1B. In addition, a list of tocopherols detected is presented in Table 2. WLEs contained α-tocopherol at concentrations of 1.3 mg/100 g of WLEs, and 0.6 mg/100 g of walnuts, and γ-tocopherol at concentrations of 82.4 mg/100 g of WLEs, and 37.1 mg/100 g of walnuts. Previously, it was reported that γ-tocopherol is the most abundant tocopherol in walnuts (19,21). Moreover, Savage and McNeil (21) reported that walnut oils contained α-tocopherol at 14.8-28.7 mg/g of and γ-tocopherol at 206.9-355.0 mg/g of oil. In particular, Vina cultivar exhibited a fatty acid composition similar to WLEs in this study with comparable concentrations of α-tocopherol (24 mg/g of oil) and γ-tocopherol (300.9 mg/g of oil).

FACS isolation of CD133⁺CD44⁺HCT116 cells CSCs have been identified based on expressions of specific cell surface makers. CD133 and CD44 are widely considered to be cancer stem/initiating cell markers (4) and, thus, CD133⁺CD44⁺HCT116 cells were isolated using FACS. Double-stained CD133⁺CD44⁺ cells represented 74.3% of cells isolated. Twenty percent of these isolated cells with the highest levels of fluorescence were designated as CD133⁺CD44⁺CSCs, whereas 20% of cells with the lowest levels of fluorescence were identified as

Table 2. Chemical compositions of tocopherols from WLEs and walnuts

Tocopherol	Content (mg/100 g of WLEs)	Content (mg/100 g of walnuts)
α-tocopherol	1.30±0.09 ¹⁾	0.6±0.04
γ-tocopherol	82.40±0.20	37.1±0.10

¹⁾Mean±standard error of the mean

Fig. 2. Isolation of CD133⁺CD44⁺HCT116 cells using Flow-Activated Cell Sorting (FACS). Expression of the CSCs markers CD133 and CD44 on the surface of HCT116 cells was detected using flow cytometry. (A) Expressions of CD133 and CD44 are shown in a 2-parameter plot. The lower-left quadrant represents cells that were negative for both CD133 and CD44. P5 indicates the lower 0.3% of cells among total cells. The upper-left quadrant includes cells that were positively stained for PE-labeled CD133 and were negative for CD44. The upper-right quadrant includes cells that were positively stained for both PE-labeled CD133 and FITC-labeled CD44. P4 indicates the upper 22.5% of cells among total cells. The lower-right quadrant includes cells positively stained for FITC-labeled CD44 and negative for CD133. (B) Cell counts for CD133-positive cells (upper panel) and cell counts for CD44-positive (lower panel) cells.

control cells (Fig. 2). Both CD44 and CD133 have been identified a putative CSC markers for colon cancer, and have been associated with aggressive cancer types and a poor prognosis (22). Previously, double-positive CD133⁺CD44⁺ cells were subcutaneously injected into nude mice, and 1x10⁴ cells were sufficient to establish a tumor (3). CD133⁺CD44⁺ cells have also been found to exhibit greater tumorigenicity than CD133⁻ or CD44⁻ cells (4). Thus, use of both markers to identify colon CSCs is more reliable than the use of either marker alone.

Effects of WLEs on the self-renewal capacity of colon CSCs CSCs are characterized by a self-renewal capacity that allows these cells to form colonies from single cells and non-adherent spheres. Moreover, the ability of CSCs to form a colony from a single cell has been shown to be directly proportional to the number of self-renewal cells present in a cell population (23). In this study, after treating CD133⁺CD44⁺HCT116 cells with different dosages of WLEs for 10 days, numbers of colonies present in each well were counted and used for calculation of the plating efficiency (Fig. 3A and 3B). WLEs significantly (p<0.05) suppressed colony formation at concentrations

Fig. 3. Effects of WLEs on the self-renewal capacity of CSCs. (A-B) Clonogenic assays were performed for CD133⁺CD44⁺HCT116 cells treated with WLEs at 0, 0.1, 0.5, and 1.0 mg/mL. After 10 days, colonies present were fixed and stained. (A) Microscopic images of colony formation were obtained (magnification, 100x) (B) Numbers of colonies counted and the plating efficiency were calculated as: (the number of colonies)/(the total number of total cells) × 100. (C-D) For sphere formation assays, CD133⁺CD44⁺HCT116 cells were treated with WLEs at 0, 0.1, 0.5, and 1.0 mg/mL in a sphere medium for 6 d. (C) Images of sphere formation were obtained (magnification, 100x). (D) Numbers of spheres were counted. More than 3 independent experiments were performed. Letter labels on the bar graph indicate values that significantly differed ($p < 0.05$) based on a one-way ANOVA and Tukey's post-hoc test. WLEs; walnut lipid extracts

of 0.5 and 1.0 mg/mL (22 and 37%, respectively) compared with controls, while 0.1 mg/mL WLEs only weakly inhibited colony formation (Fig. 3B).

Formation of anchorage-independent spheres in a serum-free medium assay is directly proportional to the number of self-renewing cells present (23). All 3 dosages (0.1, 0.5, and 1.0 mg/mL) of WLEs were found to disturb sphere formation (Fig. 3C and 3D). The greatest inhibition of sphere formation of 44% was observed in the presence of 1.0 mg/mL WLEs, which was significantly greater ($p < 0.001$) than for controls.

Several studies have examined the ability of a diverse set of bioactive substances to target CSCs. For example, the dietary components sulforaphane in cruciferous vegetables, β -carotene in carrots, curcumin in curry spices, and (–)-epigallocatechin-3-gallate (EGCG) in tea, reportedly inhibited the self-renewal capacity of CSCs (24-26). Similarly, walnuts are known to provide health benefits as application of walnut extracts to breast cancer cells resulted in a decrease in cell proliferation (27), while treatment of hippocampal cells with walnut extracts resulted in deleterious effects involving inflammation and oxidative stress (28).

Omega 3 (n-3), also known as ALA, is one of the PUFAs present in WLEs with beneficial effects on human health. Dietary ALA has been shown to be beneficial for patients with hypercholesterolemia, based on reduction of inflammation and risk factors for cardiovascular disease that were identified in lipid profiles (29). In estrogen-positive

breast cancer cells, ALA has exhibited pro-apoptotic and growth-inhibitory effects. Moreover, n-3 was recently reported to be effective for prevention and treatment of colorectal cancer, with anti-CSC effects observed in human colorectal cancer cells *in vitro* (30). Furthermore, dietary walnuts also reportedly suppressed colorectal cancer in mice, based on suppression of inflammation, vascularization, and proliferation effects, as well as apoptosis (17).

Alpha and γ -tocopherols are the main isomers of vitamin E, which is a well-known anti-oxidant nutrient from plant sources. Gamma-tocopherol mediates anti-cancer effects via anti-oxidative and anti-inflammatory mechanisms, and γ -tocopherol-rich mixed tocopherols have been shown to suppress tumorigenesis of colon cancer and related inflammatory symptoms *in vivo* (31).

Effects of WLEs on expressions of Notch1 and the Wnt signaling pathway in CSCs

The stem cell marker, Notch1 was used to determine whether WLEs suppress colon CSCs stemness (Fig. 4A). Notch signaling has been shown to play an important role in cell-fate determination, and also has important roles in cell survival and proliferation (8). Moreover, Fender *et al.* (32) recently demonstrated that Notch1 increases the stemness and enhances the epithelial-to-mesenchymal transition (EMT) in human colorectal cancer, based on comparisons between colon tumor tissues and normal colon tissues, and on studies of HCT116 colon cancer cells *in vitro*. In this study, expression of Notch1 was significantly ($p < 0.001$) suppressed in the

Fig. 4. Effects of WLEs on protein levels of Notch1 and Wnt/ β -catenin signaling in colon CSCs. Protein levels of (A) Notch1, (B) β -catenin, and (C) p-GSK3 β were examined using Western blotting assays in CD133⁺CD44⁺HCT116 cells. α -Tubulin was used as a loading control. More than 3 independent experiments were performed. Letter labels on the bar graph indicate values that significantly differed ($p < 0.05$) based on a one-way ANOVA and Tukey's post-hoc test. WLEs; walnut lipid extracts

presence of WLEs in a dose-dependent manner, compared with control cells. In addition, down regulation of Notch1 by WLEs was associated with suppression of CSC maintenance. WLEs are also rich in n-3 fatty acid, and n-3 eicosapentaenoic acid was previously shown to decrease CD133 colon cancer stem-like cells, based on up-regulation of the differentiation markers cytokeratin 20 and mucin 2, and by down-regulation of CD133 expression (33). Thus, WLEs probably affect colon CSCs stemness by down-regulation of stem cell marker Notch1 expression.

To investigate the mechanism(s) by which WLEs regulate the self-renewal capacity of colon CSCs, expressions of β -catenin and phosphogluco-kinase 3 β (p-GSK3 β), which represent targets in the Wnt/ β -catenin signaling, were examined. The Wnt/ β -catenin signaling pathway is involved in transformation of normal colonic epithelial cells and plays a pivotal role in promotion of the self-renewal capacity of CSCs, and also defines CSCs. In the cytoplasm, β -catenin is phosphorylated by GSK3 β to trigger its ubiquitination. Translocation of β -catenin to the nucleus and accumulation of phosphorylated GSK3 β leads to activation of the Wnt/ β -catenin signaling pathway, and to stem cell maintenance and self-renewal (34). WLEs treatments significantly ($p < 0.001$) suppressed both expression of β -catenin and phosphorylation of GSK3 β in a dose-dependent manner, compared with controls (Fig. 4B and 4C). Reduced expression of these markers suggests that effects of WLEs on the self-renewal capacity of CD133⁺ CD44⁺HCT116 cells is

mediated via the Wnt/ β -catenin signaling pathway.

WLE contains multiple components, including n-3, n-6, PUFAs, MUFAs, SFAs, and tocopherols. The anti-CSCs effects of the individual components of WLE were not investigated in this study. Anti-CSCs effects of WLEs may be due to the presence of n-3 fatty acids, γ -tocopherols, and other nutrients in combination. Therefore, future investigation of whether individual components of WLEs or combinations of WLEs components mediate the anti-CSCs effects observed will be important. Walnuts also contain plant sterols in a concentration range from 90–283 mg/100 g (19). Several phytosterols, including β -sitosterol, fucosterol, and gramisterol, have reportedly showed anti-proliferative effects in leukemia (35). However, the composition of phytosterols was not analyzed in this study. The anti-CSCs effect of phytosterol in walnuts remains to be investigated. In conclusion, WLEs containing combinations of essential fatty acids and γ -tocopherol mediated therapeutic effects against recurrent CSCs.

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