

Full Paper

Sesame lignans upregulate glutathione S-transferase expression and downregulate microRNA-669c-3p

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Oxidative stress is associated with aging and pathologies such as cardiovascular diseases, Alzheimer's disease, and cancer. Glutathione S-transferase (GST), a family of detoxification enzymes, plays a crucial role in countering oxidative stress. Therefore, there is a need for the development of physiologically functional foods and agricultural products, which enhance GST activity. Sesamin and episesamin are major lignans in refined sesame oil that exhibit beneficial properties including antioxidative stress effects. A previous study showed that sesamin upregulated GST activity. This study aimed to elucidate the mechanism underlying the GST activity enhancement elicited by sesame lignans. C57BL/6J mice were orally administered 20 mg/kg body weight sesame lignans (sesamin:episesamin=1:1) for 7 days. Oral administration of sesame lignans increased the GST activity in the mouse liver. Furthermore, the lignans upregulated GSTA1, GSTA4, and GSTM4 protein expression. Microarray analysis revealed that sesame lignans changed the expression of various microRNAs (miRNAs) (84 upregulated, 19 downregulated). We also found 16 miRNAs, including miR-669c-3p, that may negatively regulate GST expression among the 19 miRNAs with reduced expression caused by the sesame lignans. miR-669c is reportedly negatively correlated with GST. Additionally, we transfected NMuLi cells with an miR-669c-3p mimic and evaluated the effect of miR-669c-3p on GST mRNA and protein expressions. The results showed that the miR-669c-3p mimic suppressed the mRNA and protein levels of GSTA4 and GSTM4. In conclusion, sesame lignans increased GST protein expression and activity and downregulated miRNAs, including miR-669c-3p, which is a possible suppressor of GST.

Key words: sesame lignans, sesamin, episesamin, glutathione S-transferase, microRNA

INTRODUCTION

Oxidative stress is defined as an imbalance between increased levels of reactive oxygen species and decreased antioxidant activity. Increased oxidative stress damages lipids, proteins, and DNA. This oxidative damage is commonly associated with aging [1] and pathologies such as cardiovascular diseases, Alzheimer's disease, and cancer [2–4].

Glutathione S-transferase (GST) comprises a family of detoxification enzymes that protect against oxidative stress [5]. The GST family includes the GST isoform A (GSTA), GST isoform M (GSTM), and others. GSTA1 and GSTA2 regulate the concentrations of lipid peroxidation (LPO) products [6]. GSTA4 is involved in the accumulation of 4-hydroxynonenal (4-HNE) and survival under oxidative stress [7, 8]. Additionally, in studies on accelerated aging, GST activity was decreased in the plasma and liver of mice [9], suggesting that increased GST activity impedes aging and the development of diseases.

Sesamin is one of the main lignans in sesame seeds. It is partially isomerized to episesamin during the refining process in sesame oil production. Sesame lignans exhibit various biological properties, such as antioxidative, anti-inflammatory effects [10, 11]. Antioxidative effects of sesame lignans result in protective properties against liver injury, cardiovascular disease, and cognitive decline [12–14]. In the rabbit liver, sesame lignans decreased the level of 4-HNE adduct protein, the main end product of peroxidation in lipids [12]. A previous study demonstrated that sesamin upregulated GST activity and exerted a suppressive effect against exercise-induced plasma LPO in mice [15]. However, the mechanism through which sesamin activates GST remains poorly understood.

MicroRNAs (miRNAs) are small noncoding RNAs that regulate target genes by mRNA destabilization or translational inhibition [16]. Recent studies have reported that miRNAs play a crucial role in various biological processes, including cell proliferation, apoptosis, metabolism, and inflammation [17–20].

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Several studies have demonstrated that miRNAs are one of several types of oxidative stress regulator [21, 22].

This study aimed to elucidate the effects of sesame lignans on GST activity and miRNA expression in the mouse liver. We orally administered sesame lignans to mice and assessed GST activity and expression. Moreover, we performed a comprehensive analysis of miRNA expression profiles and evaluated the effects of miRNA on GST expression.

MATERIALS AND METHODS

Chemicals and materials

The mixture of sesamin and episesamin (sesamin:episesamin=1:1, SE) was purchased from Takemoto Oil & Fat (Nagoya, Japan). Purified olive oil was purchased from Nacalai Tesque (Kyoto, Japan). Pierce BCA protein assay kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). A GST assay kit was purchased from Cayman Chemical (Ann Arbor, MI, USA). An miRNeasy kit was purchased from QIAGEN (Hilden, Germany). mirVana™ miRNA Mimic Negative Control #1 and an mmu-miR-669c mirVana™ miRNA mimic were purchased from Ambion (Austin, TX, USA). TaqMan probes (Gstm4, Gapdh, mmu-miR-669c-3p, U6) were purchased from Applied Biosystems (Foster City, CA, USA). Anti-GSTA1, anti-GSTM4, and anti-GAPDH antibodies were purchased from Abcam (Cambridge, UK). Anti-GSTA4 antibody was purchased from Proteintech Group (Chicago, IL, USA).

Animals

Eleven-week-old male C57BL/6J mice (Kyudo Co., Ltd., Saga, Japan) were maintained under an L12:D12 photoperiod in an air-conditioned room (20°C and 60% relative humidity). Protocols for animal care and experiments were approved by the Animal Care and Use Committee of Kyushu University. SE (20 mg/kg body weight/day), dissolved in purified olive oil, was orally administered at 24 hr intervals for 7 days. Purified olive oil was administered to the control group on the same schedule. Mice were anesthetized with isoflurane vapor for blood collection and subsequently euthanized. Livers were excised and stored at -80°C. We performed microarray and *in vivo* analyses on the same samples (control group, n=12; SE group, n=12).

Cell culture

NMuLi cells (ATCC, Manassas, VA, USA), derived from a normal mouse liver, were maintained in Dulbecco's modified eagle's medium (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA). NMuLi cells were cultured at 37°C in a humidified chamber with 5% CO₂.

RNA transfection

RNA reagents were introduced into cells with Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA). RNA reagents, RNAiMAX, and Opti-MEM™ I Reduced Serum Medium (Gibco, Waltham, MA, USA) were gently mixed by pipetting. After an additional 10 min at room temperature, the complexes were added to the NMuLi cells and cultured for 48 or 72 hr.

Measurement of enzymatic activity in the liver

Liver tissues were homogenized in ice-cold Pi buffer (10 µL/mg tissue) with a protease and phosphatase inhibitor cocktail (100×; Thermo Fisher Scientific, Waltham, MA, USA). The homogenates were then centrifuged at 10,000 × g for 20 min, and the GST activity of the supernatants diluted to 1/50 was measured with the GST assay kit. Results were normalized to the amount of total protein measured using the Pierce BCA protein assay kit.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the liver using the miRNeasy kit, and cDNA was synthesized from 2 µg of total RNA using a high-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA, USA) or 50 µg of total RNA using a TaqMan microRNA reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Total RNA was extracted from cells using TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA), and cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara Bio Inc., Siga, Japan). Gene expression was then measured using a StepOnePlus (Applied Biosystems, Waltham, MA, USA) or CFX384 Touch™ Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For the RNA assay, TaqMan fast universal polymerase chain reaction (PCR) master mix (Applied Biosystems, Waltham, MA, USA) and TaqMan probes, iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers (Table 1), or SSoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and primers (Table 1) were added to cDNA. For the miRNA assay, TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA) and TaqMan probes were added to the cDNA. Gene expression was normalized to Gapdh expression. The results are presented relative to the average of the control group, which was set as 1. MicroRNA expression was normalized to U6 expression.

Immunoblotting

Liver tissue was homogenized in RIPA buffer with protease and phosphatase inhibitor. Cells were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM ethylenediamine tetra-acetic acid, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1 mM phenylmethanesulfonyl fluoride, 2 mg/mL aprotinin, and 1 mM pervanadate). Then, 2× sample buffer solution (Bio-Rad Laboratories, Hercules,

Table 1. Primer sequences

| Gene | Forward | Reverse |
|-------|-----------------------------------|----------------------------------|
| Gsta1 | 5'- CCCCTTCCCTCTGCTGAAG-3' | 5'- TGCAGCTTCACTGAATCTTGAAAG-3' |
| Gsta4 | 5'-AACTTGATGGGAAGGACCTGAA-3' | 5'-CCACGGCAATCATCATC-3' |
| Gstm4 | 5'-ATCACGCAGAGCAATGCCATCC-3' | 5'-GGAGACATCCATAGCCTGGTTC-3' |
| Gapdh | 5'-CGACTTCAACAGCAACTCCCACTCTCC-3' | 5'-TGGGTGGTCCAGGGTTCTTACTCCTT-3' |

CA, USA) with 0.01% (w/v) mercaptoethanol or Laemmli sample buffer (0.1 M Tris-HCl buffer, pH 6.8, 1% SDS, 0.05% mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) was added and heated at 95°C for 5 min. Samples were separated by reducing 10% (weight/volume) polyacrylamide gel electrophoresis and electroblotted on polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) or Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated for 1 hr in PVDF blocking buffer (Toyobo Co., Ltd., Osaka, Japan) or Tween 20/tris-buffered saline containing 1% bovine serum albumin and incubated overnight in the presence of either anti-GSTA1, anti-GSTA4, anti-GSTM4, or anti-GAPDH primary antibodies (1:2,000–5,000). After subsequent incubation for 60 min at 25°C in the presence of an HRP-conjugated secondary antibody (rabbit antibody, 1:10,000–20,000; GE Healthcare, Menlo Park, CA, USA, or Cell Signaling Technology, Danvers, MA, USA; goat antibody, 1:10,000–20,000; Abcam or eBioscience), complexes were visualized by chemiluminescence. GSTA1, GSTA4, and GSTM4 protein levels were normalized to GAPDH level. The results are presented relative to the average of the control group, which was set as 1.

Microarray analysis

Total RNAs in the liver were extracted with TRI reagent (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy MinElute Kit (QIAGEN, Hilden, Germany). Twelve samples of the same group were pooled into four samples each, resulting in three sample categories. Fluorescent labeling of RNA samples was performed using a 3D-Gene® miRNA labeling kit (Toray Industries, Tokyo, Japan). Expression levels of miRNA were measured using DNA chips (3D-Gene Mouse miRNA Oligo chip ver.21 [Mouse_miRNA_V21], Toray Industries). MicroRNA target sites within a UTR were predicted with the TargetScan software available at http://www.targetscan.org/mmu_72/.

Statistics

Results are expressed as the mean ± standard error of the mean. The Western blotting data of one sample for GSTA4 were excluded because of measurement failure. Data were analyzed using a Student's *t*-test for two-group comparisons. *P* values <0.05 were considered statistically significant.

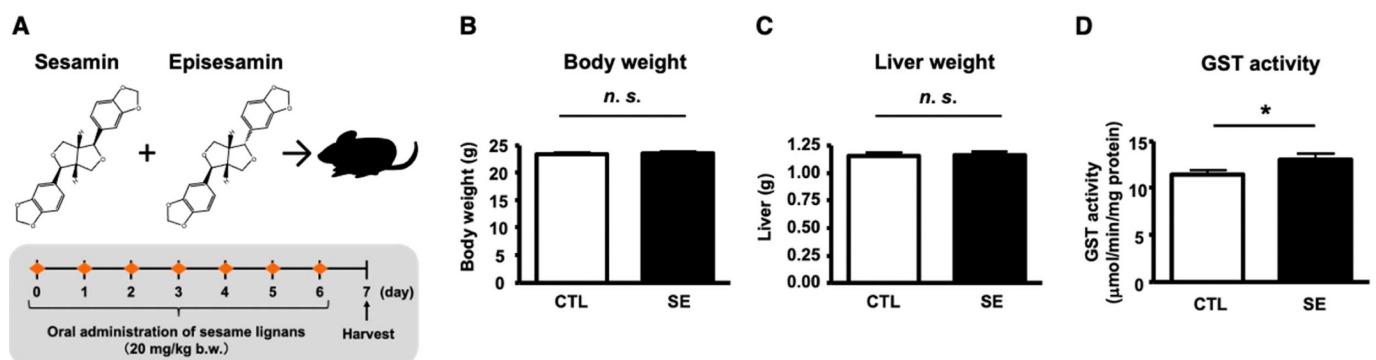


Fig. 1. Effect of sesame lignans on glutathione S-transferase (GST) activity in the mouse liver.

Sesame lignans (SE, 20 mg/kg body weight) were orally administered to C57BL/6J mice for 7 days. (A) Sesame lignan oral administration scheme. (B) Final body weight and (C) liver weight. (D) GST activity in liver homogenates measured with the GST assay kit. Data are presented as the mean ± SEM (*n*=12). **p*<0.05 by two-tailed Student's *t*-test. CTL: control; n.s.: not significant.

RESULTS

Oral sesame lignan intake upregulates GST activity in the liver

GST plays a crucial role in the protection against oxidative injury. In a previous study, sesame lignans upregulated GST activity and exhibited protective effects against oxidative stress in the liver [15]. To assess the effect of sesamin and episesamin on GST activity in the liver, 20 mg/kg body weight SE was orally administered to C57BL/6J mice for 7 days (Fig. 1A). The human equivalent dose for 20 mg/kg body weight SE is 1.63 mg/kg body weight, which corresponds to a 97.6 mg/person of SE for a person weighing 60 kg (calculated using a formula for dose translation based on surface area) [23]. In a previous clinical study, subjects received 100 mg/person/day sesame lignans for 7 days, and no severe adverse effects were reported [24]. The study reported that 100 mg/person/day sesame lignan intake suppressed the exercise-induced increase of plasma lipid peroxide levels [24].

No significant differences were observed between the control (CTL) and SE groups in body and liver weight (Fig. 1B–C). Liver tissues were harvested and homogenized, and GST activity was evaluated using a GST assay kit. Oral SE intake upregulated the GST activity in the liver (Fig. 1D; *p*<0.05). Our results show that SE intake significantly upregulated GST activity in the mouse liver without changing either the body or liver weight.

Sesame lignans increase GST protein expression in the liver

GST plays a crucial role in the protection against LPO-induced oxidative stress. In this study, we assessed the GST gene expression level. Real-time PCR analysis revealed no change in GSTA1, GSTA4, and GSTM4 mRNA expression levels after oral administration of SE (Fig. 2A). By contrast, administration of SE upregulated GSTA1, GSTA4, and GSTM4 protein expression (Fig. 2B; *p*<0.05, *p*<0.01, *p*<0.001). These results indicate that SE increased GST activity via the upregulation of GST proteins.

Sesame lignans downregulate miR-669c-3p expression in the liver

MicroRNAs destabilize mRNA or inhibit translation by binding to the 3'-untranslated region of target genes. Numerous studies have reported that miRNAs are involved in the physiological properties of foods [25, 26]. However, little is known about the effect of sesame lignans on hepatic miRNAs

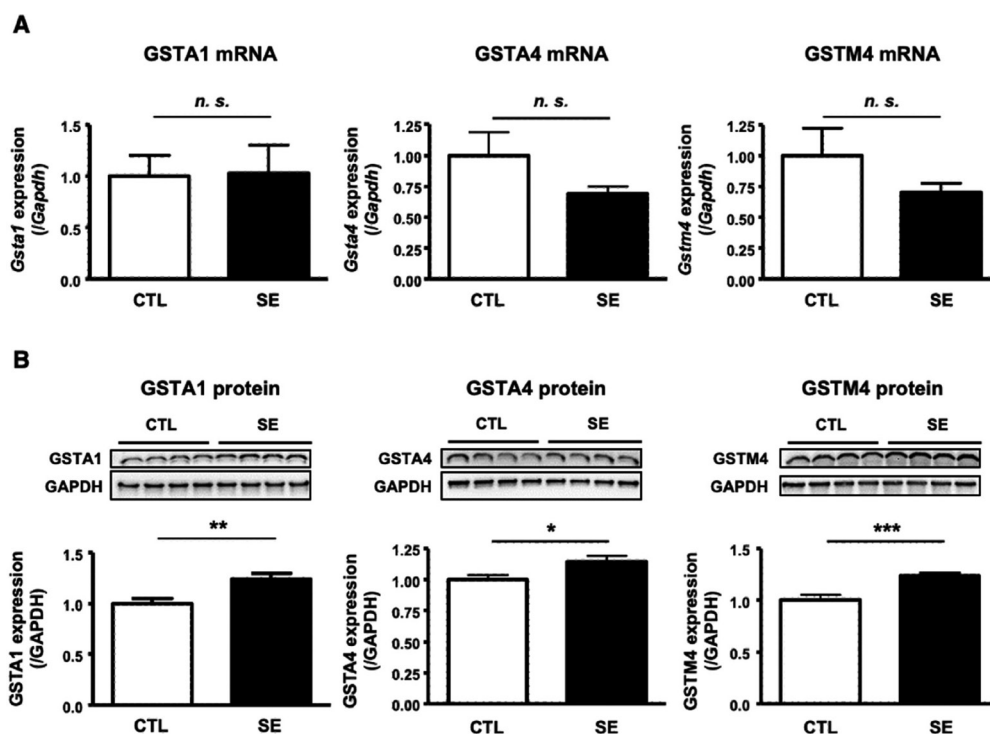


Fig. 2. Sesame lignans increase glutathione S-transferase (GST) protein expression in the mouse liver.

Sesame lignans (SE, 20 mg/kg body weight) were orally administered to C57BL/6J mice for 7 days. (A) Total mRNA was isolated from the liver, and GST mRNA expression was measured by quantitative real-time PCR. (B) GST protein expression was evaluated by Western blotting. The experimental unit in this study was the individual mouse. Data are presented as the mean ± SEM ($n=11-12$). * $p<0.05$ by two-tailed Student's *t*-test. ** $p<0.01$ by two-tailed Student's *t*-test. *** $p<0.001$ by two-tailed Student's *t*-test. CTL: control; n.s.: not significant.

expressions. To examine the effect of SE on miRNAs in the liver, we conducted an miRNA microarray analysis of liver tissue from mice administered SE (Fig. 3A). SE upregulated the expression of 84 miRNAs and downregulated the expression of 19 miRNAs in the liver (Fig. 3B, C; fold change <0.85 or >1.15 ; $p<0.25$). To identify miRNAs that may target GST, we used miRNA target prediction algorithms (TargetScan). The results showed that 16 of the 19 miRNAs with downregulated expression caused by SE may target GST (Fig. 3C, D). A previous study reported that miR-669c expression was negatively correlated with GST expression [27]. We confirmed the effect of SE on miR-669c-3p by quantitative real-time PCR. Consistent with microarray data, quantitative real-time PCR analysis demonstrated that SE increased the average ΔCt (Ct [miR-669c-3p] - Ct [U6]). In other words, they decreased miR-669c-3p expression in the mouse liver (Table 2). These results show that SE downregulated various miRNAs, including miR-669c-3p, a possible suppressor of GST.

miR-669c-3p suppresses GST protein expression

Our database analysis (TargetScan) revealed that miR-669c-3p can suppress GST expression (Fig. 3D). We assessed whether miR-669c-3p decreased GST mRNA and protein expression in NMuLi cells derived from a normal mouse liver. The transfection of an miR-669c-3p mimic suppressed GSTA1, GSTA4, and GSTM4 mRNA expression (Fig. 4A; $p<0.01$, $p<0.001$). Also, the miR-669c-3p mimic downregulated GSTA4 and GSTM4 protein expression but did not change GSTA1 protein expression (Fig. 4B; $p<0.05$, $p<0.001$). These results suggest that miR-669c-3p

downregulated the mRNA and protein levels of GSTA4 and GSTM4 in the mouse liver.

DISCUSSION

Oxidative stress is involved in aging and pathologies such as cardiovascular diseases, Alzheimer's disease, and cancer. Tripeptide glutathione (GSH) is present in all mammalian tissue and is the most abundant nonprotein thiol that protects against oxidative stress [28]. GST plays a crucial role in cellular detoxification and protects cells against reactive oxygen metabolites via the conjugation of GSH with numerous substrates, including hydroperoxide [29]. Overexpression of GSTA4 in HepG2 cells upregulates resistance to oxidative stress [30]. Consistent with these conditions, GSTA4 null mice exhibited decreased survival under oxidative stress compared with wild-type mice [8]. Polymorphisms of GST, particularly GSTT1, enhance the lung effects of exposure to tobacco smoke [31]. Additionally, several studies have reported that GST gene polymorphisms were related to an increased risk of developing alcoholic liver disease [32, 33]. These findings suggest that GST activation could be an ideal countermeasure against aging and disease.

Sesamin and episesamin are lignans found in refined sesame oil that exhibit various biological properties. For example, they have been reported to suppress oxidative stress [10, 12, 15]. However, sesamin and episesamin do not exhibit strong radical scavenging properties *in vitro* [34, 35]. Sesamin is converted to SC1 (mono-

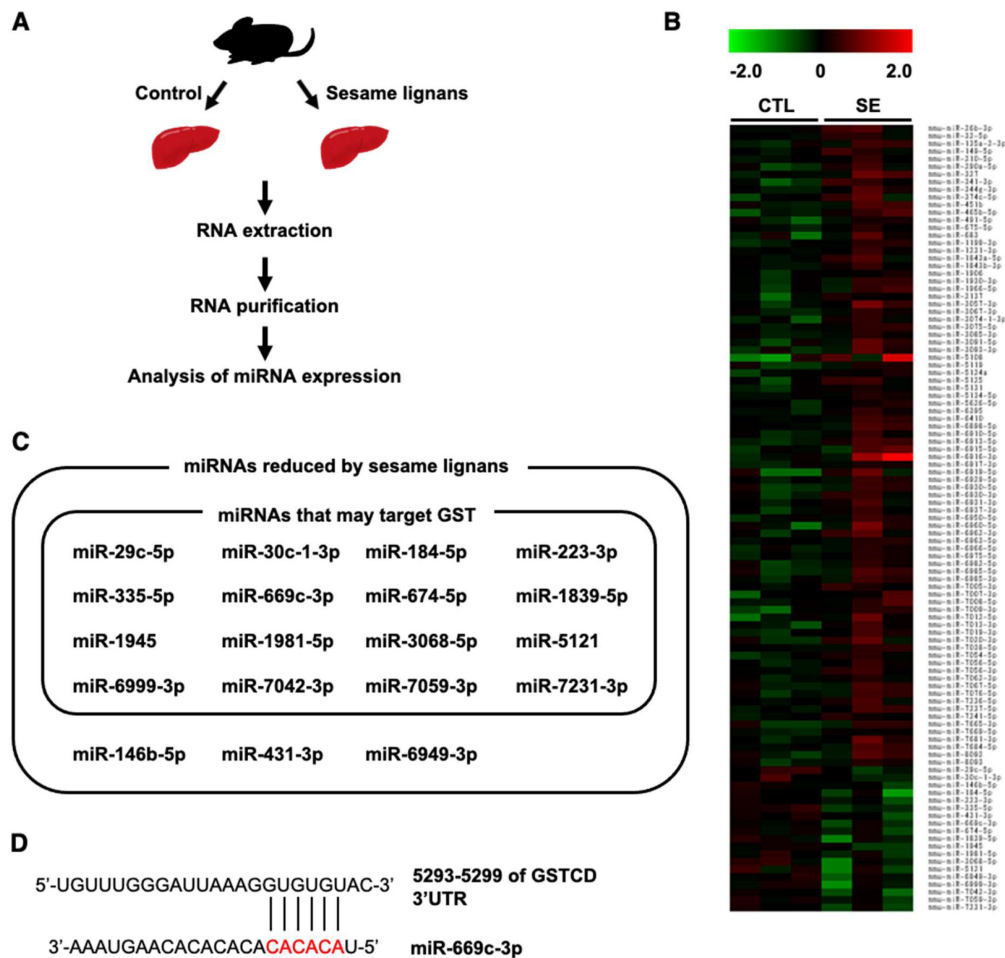


Fig. 3. Comprehensive analysis of miRNA expression affected by sesame lignans.

Sesame lignans (SE, 20 mg/kg body weight) were orally administered to C57BL/6J mice for 7 days. (A) Microarray analysis of miRNAs expression after purification of small RNAs in the mouse liver. (B) Heatmap of miRNAs (fold change <0.85 or >1.15 ; $p<0.25$). (C) miRNAs with downregulated expression caused by the sesame lignans. (C, D) Results of TargetScan, which was used to search for miRNAs that target GST. CTL: control.

Table 2. Level of miR-669c-3p expression in the liver measured by quantitative RT-PCR

| | Average Ct ^a (U6) | Average Ct ^a (miR-669c-3p) | The average of ΔCt^b (Ct(miR-669c-3p) – Ct(U6)) |
|-----|---------------------------------|--|--|
| CTL | 22.74 \pm 0.11 | 31.28 \pm 0.12 | 8.54 \pm 0.13 |
| SE | 22.54 \pm 0.06 | 31.54 \pm 0.15 | 9.00 \pm 0.17* |

CTL: control; SE: sesame lignans.

^aCt is the number of cycles that fluorescence passes the threshold in the amplification curve.

^b $\Delta Ct = Ct(miR-669c-3p) - Ct(U6)$. Data are presented as the mean \pm SEM ($n=12$). * $p<0.05$ by two-tailed Student's *t*-test.

catechol metabolite of sesamin) in the liver; similarly, episesamin is converted to EC1 (a mono-catechol metabolite of episesamin) in the liver [36]. These major catechol metabolites exhibit radical scavenging properties that are stronger than those of sesamin and episesamin [37]. However, these antioxidative activities were evaluated by using sesame lignans metabolites at mM concentrations [37]. In a human study, the plasma concentrations of SC1 and EC1 reached a peak at 5.0 hr, and the maximum concentrations were 187 ± 75 ng/mL (SC1) or 88 ± 35 ng/mL (EC1) after 50 mg sesame lignan intake [36]. Therefore, the anti-

oxidative effect of sesamin and episesamin has some mechanisms other than the radical scavenging properties of these metabolites.

A recent study reported that sesame lignans activate GST, which plays a crucial role in oxidative stress tolerance [15]. Consistent with the previous findings, our data show that 20 mg/kg body weight SE intake upregulated GST activity. The human equivalent dose for 20 mg/kg body weight SE is 1.63 mg/kg body weight, which corresponds to a 97.6 mg dose of SE for a person weighing 60 kg (calculated using a formula for dose translation based on surface area) [23]. In a previous clinical study, subjects

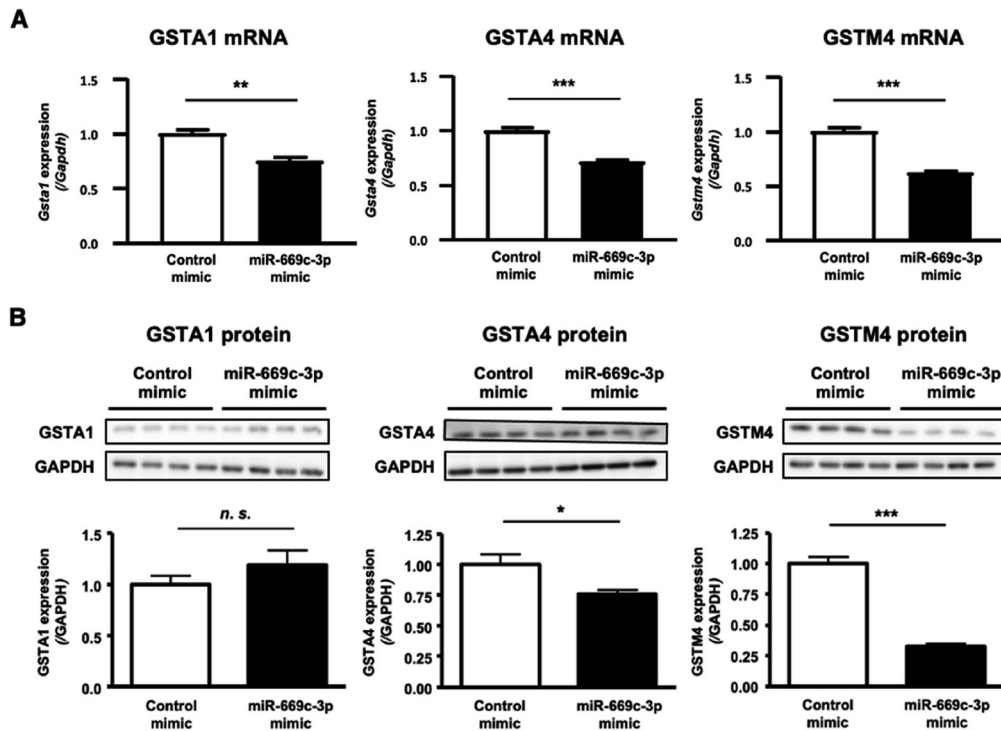


Fig. 4. miR-669c-3p downregulates glutathione S-transferase (GST) protein expression.

(A, B) Expression of GST mRNA (A) and protein (B) in NMuLi cells transfected with 20 nM miR-669c-3p mimic for 48 or 72 hr and evaluated by quantitative real-time PCR or Western blotting. Data are presented as the mean \pm SEM ($n=4$). * $p<0.05$ by two-tailed Student's *t*-test. ** $p<0.01$ by two-tailed Student's *t*-test. *** $p<0.001$ by two-tailed Student's *t*-test. n.s.: not significant.

received a 100 mg/person/day sesame lignan supplement for 7 days, and no severe adverse effects were reported [24]. The study reported that 100 mg/person/day sesame lignan intake suppressed the exercise-induced increase of plasma lipid peroxide levels [24]. These results suggest that SE can upregulate GST activity in humans.

Among the various isoforms of GST, GSTA and GSTM are abundant in mammals. In this study, we assessed the effects of SE on the expression of GSTA and GSTM. SE did not affect mRNA expression but did increase GST protein expression.

SE upregulated GSTA1, GSTA4, and GSTM4 protein expression. Sesame lignans have been reported to decrease the LPO induced by exercise and 4-HNE adduct protein induced by a fat/cholesterol-enriched diet [11, 12]. LPO and 4-HNE are substrates of GSTA1 and GSTA4, respectively [6–8]. Previous studies showed that GSTA1 catalyzed GSH-dependent LPO reduction [6] and that 4-HNE accumulates in the liver of GSTA4 null mice [8]. GSTA4 decreases 4-HNE adducts and increases survival under oxidative stress induced by paraquat or hydrogen peroxide [7]. Sesamin intake also improves resistance to paraquat or hydrogen peroxide in *Caenorhabditis elegans* [38]. These findings indicate the possibility that SE decrease oxidation products of lipids through GSTs and result in resistance to oxidative stress.

An miRNA is a small noncoding RNA that regulates target gene expression. It plays a crucial role in biological processes including cell proliferation, apoptosis, metabolism, and inflammation. It is also one of the regulators of oxidative stress, such as in the case of miR-223, which ameliorates alcoholic liver

injury by inhibiting an oxidative stress pathway [21]. Dietary factors, including polyphenols, have been shown to change miRNA gene expression [25, 26]. We evaluated the effects of SE intake on miRNA expression in the mouse liver. Our results show that SE changed various miRNA expressions, including miR-669c-3p (84 miRNAs upregulated; 19 miRNAs downregulated). Additionally, we assessed the effect of sesamin or episesamin on miR-669c-3p and GST expression in NMuLi cells. These lignans did not change miR-669c-3p and GST expression (data not shown). Since the liver consists of various cells, including hepatocytes and nonparenchymal cells, it is difficult to evaluate the effect of sesame lignans on miRNA and GST in an *in vitro* test. We also evaluated the effect of miR-669c-3p on GST mRNA and protein expression. The transfection of an miR-669c-3p mimic downregulated GSTA4 and GSTM4 expression. These data suggested that miR-669c-3p is involved in the increase of GSTA4 and GSTM4 protein expression caused by SE intake. However, the miR-669c-3p mimic did not affect GSTA1 protein expression. Therefore, additional miRNAs (Fig. 3C) may be involved in the upregulation of GSTA1 caused by SE.

In summary, this study revealed that SE upregulated GST activity and reduced miR-669c-3p, which can target GST in the mouse liver. These data constitute a crucial step in deepening our understanding of the relationship between SE and miRNAs.

CONFLICTS OF INTEREST

The authors declare no competing financial interest.

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