Dark-colored maple syrup treatment induces S-phase cell cycle arrest via reduced proliferating cell nuclear antigen expression in colorectal cancer cells

TETSUSHI YAMAMOTO, TOMOYO NISHITA and ATSUSHI TAGA

Pathological and Biomolecular Analyses Laboratory, School of Pharmacy, Kindai University, Higashi-Osaka, Osaka 577-8502, Japan

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Abstract. Maple syrup is a natural sweetener that is consumed worldwide. It has been previously reported that dark-colored maple syrup exerts an inhibitory effect on colorectal cancer (CRC) proliferation and invasion. In the present study, the underlying mechanism of CRC cell growth inhibition was examined with dark-colored maple syrup treatment using a shotgun liquid chromatography-tandem mass spectrometry-based global proteomic approach. Applying a semi-quantitative method based on spectral counting, 388 proteins were identified with expression changes of >1.5-fold following dark-colored maple syrup treatment. Gene Ontology analysis revealed that these proteins possessed cell cycle-associated functions. It was also indicated that CRC cells treated with dark-colored maple syrup exhibited decreased proliferating cell nuclear antigen (PCNA) expression and S-phase cell cycle arrest. Dark-colored maple syrup treatment also resulted in altered expression of cell cycle-associated genes, including cyclin-dependent kinase (CDK)4 and CDK6. In conclusion, these data suggested that dark-colored maple syrup treatment induced S-phase cell cycle arrest in CRC cells by reducing the expression of PCNA and regulating cell cycle-associated genes. These findings suggest that dark-colored maple syrup may be a source of compounds for the development of novel drugs for colorectal cancer treatment.

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed types of cancer and is the second leading cause of cancer-associated mortality worldwide (1,2). Etiological studies report that dietary factors serve an important role in CRC carcinogenesis (3-5). CRC risk is increased by a high intake of red and processed meats (6), as red meat is a contributing factor in the initiation of colorectal carcinogenesis (7). However, milk and other dairy products reportedly have a protective effect against CRC, due to their high calcium content and bioactive constituents, including vitamin D (8-10). Fermented dairy products, including yogurt, also exhibit protective effects against CRC, possibly due to lactic acid bacteria and their reported ability to inactivate intestinal carcinogens and therefore reduce CRC risk (11). Although the present epidemiological evidence is insufficient (12-16), diet-associated preventative measures may be an important strategy for CRC reduction.

Maple syrup is a natural sweetener produced by boiling down sap, which is collected from the sugar maple, Acer saccharum, and is consumed worldwide by individuals of all ages (17,18). The sugar maple is distributed throughout North America, and maple trees serve an important role in traditional medicine among Native Americans (19). A number of previous studies have examined the chemical composition and biological properties of maple-derived products, including maple syrup (20-26).

The climatic conditions during production season influence maple sap composition, including the color, the aroma and the taste of the maple syrup, which vary based on the season of sap collection (27-29). Maple syrup is primarily graded according to its flavor and transmittance, including visual color differences, ranging from light-colored and delicately flavored, to dark-colored and strongly flavored (18). Although the variation in composition may further lead to different grades of maple syrup, along with different biological effects, the differences in composition of ingredients among each grade of maple syrup remain unknown and require further investigation. In our previous study of the anticancer effects of different grades of maple syrup, it was reported that dark-colored maple syrup reduced AKT, also termed protein kinase B, activation, and...
therefore significantly inhibited proliferation and invasion in CRC cells (30). In addition, another previous study showed that it significantly inhibited growth in other types of gastrointestinal cancer cell (31). This suggests that dark-colored maple syrup may be a useful dietary factor for potentially preventing cancer progression.

In the present study, a shotgun liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based global proteomic analysis was performed on human CRC cells treated with different grades of maple syrup, in order to examine the underlying mechanism behind dark-colored maple syrup inhibiting CRC proliferation. Two types of maple syrup, which indicated the strongest and weakest anticancer effects in our previous study of colon cancer cells (30), were selected. A total of 388 proteins were identified that were differentially expressed in CRC cells treated with dark-grade maple syrup compared with extra-light grade maple syrup. The current study focused on the expression of proliferating cell nuclear antigen (PCNA), which is a key factor of cell cycle regulation. Therefore, further investigations were conducted on whether changes of PCNA expression following dark grade maple syrup treatment may be involved in cell cycle regulation in human CRC cells.

Materials and methods

Materials. Urea was purchased from GE Healthcare Life Sciences (Little Chalfont, UK), and thiourea and Triton X-100 were obtained from Nacalai Tesque, Inc., (Kyoto, Japan). All other chemicals and reagents were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Maple syrups were purchased at a local grocery store in Osaka, Japan, in March 2015.

Based on Canadian standards, maple syrup is classified into the following five grades: AA, extra light; grade A, light; grade B, medium; grade C, amber, and grade D dark. Since the present study was performed the maple syrup classification has changed according to the following: Golden, delicate Taste; amber, rich Taste; dark, robust taste; and very dark, strong taste (32). In Japan, these new grading maple syrup grades have been used since April 2017. Since the differences in ingredient composition among each grade of maple syrup are not yet fully understood, in the present study, two grades of maple syrup were selected: The extra light grade maple syrup, which has a slightly golden tint and a delicate flavor with >75% light transmission, and the dark grade maple syrup, which has a much darker brown color and a strong flavor with <25% light transmission.

The colorectal cancer DLD-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). All cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an atmosphere containing 5% CO₂.

Protein preparation. The DLD-1 cells were plated at a density of 2x10⁵ cells/60-mm dish with RPMI-1640 medium. The next day, the culture medium was replaced with culture medium with 1% (v/v) extra light grade maple syrup (extra), dark grade maple syrup (dark) or without syrup (control). This concentration was selected due to results from our previous study indicating a lack of cytotoxic effects against DLD-1 cells due to the high concentration of sucrose (30). After 72 h, the cells were solubilized in urea lysis buffer (7 M urea, 2 M thiourea, 5% 3-[3-Cholamidopropyl]dimethylammonio]propanesulfonate and 1% Triton X-100), and the protein concentration was measured with the Bio-Rad Protein assay (cat. no. 5000006JA; Bio-Rad Laboratories Inc., Hercules, CA, USA), according to the manufacturer's protocols.

Gel-free digestion was subsequently performed, as described previously (33). Briefly, 10 µg protein extract from each sample was reduced by adding 45 mM dithiothreitol and 20 mM tris (2-carboxyethyl) phosphine. The proteins were subsequently alkylated with 100 mM iodoacetic acid. Following alkylation, the samples were digested at 37°C for 24 h using MS-grade trypsin gold (Promega Corporation, Madison, WI, USA). Finally, the digests were purified using PepClean C-18 Spin Columns (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis for protein identification. Peptide samples (~2 µg) were injected into a peptide L-trap column (Chemicals Evaluation and Research Institute, Tokyo, Japan) with an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The samples were subsequently further separated through a Paradigm MS4 (AMR Inc., Tokyo, Japan) with a reverse-phase C18-column (L-column, 3-µm-diameter gel particles, 120 Å pore size, 0.2x150 mm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The column flow rate was 1 µl/min, and the mobile phase consisted of 0.1% formic acid in water (solution A) and acetonitrile (solution B), with a concentration gradient of 5% solution B to 40% solution B over 120 min. Gradient-eluted peptides were introduced into the mass spectrometer through the nanoelectrospray ionization (NSI) interface that had a separation column outlet directly connected with an NSI needle. The peptides were analyzed with an LTQ ion-trap mass spectrometer (Thermo Fisher Scientific, Inc.). No sheath or auxiliary gas was used. The MS scan sequence used was full-scan MS in the normal/centroid mode and sequential MS/MS in the normal/centroid mode. The positive ion mass spectra were acquired by a data-dependent manner, with MS/MS fragmentation performed on the two most intense peaks of every full MS scan with an isolation width of 1.0 m/z and a collisional activation amplitude of 35% in the m/z range of 300-2,000.

All MS/MS spectral data were searched against the SwissProt Homo Sapiens database (https://www.uniprot.org/) using Mascot version 2.4.01 (Matrix Science, Ltd., London, UK). The search criteria were ‘enzyme’ and ‘trypsin’, with the following allowances: ≤2 missed cleavage peptides; mass tolerance, ± 2.0 Da; MS/MS tolerance, ± 0.8 Da; cysteine carbamidomethylation; and methionine oxidation modifications.

Semi-quantitative analysis of identified proteins. The fold-change in expression was calculated as the log2 ratio of protein abundance (Rsc), evaluated by spectral counting (34). For comparisons, the relative amounts of identified proteins were calculated using the normalized spectral abundance factor (NSAF) (35). Differentially expressed proteins were
considered significant when the Rsc was >0.585 or <-0.585, corresponding to fold-changes of >1.5 or <0.66, respectively.

**Gene ontology (GO) analysis.** The functions of proteins that indicated altered expression with maple syrup treatment were additionally investigated. Their sequences were assigned to Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/kegg_ja.html) signaling pathway terms to examine their functional annotations using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 (http://david.abcc.ncifcrf.gov/home.jsp) (36-38). P<0.05 was considered to indicate a significant category.

**Western blot analysis.** Total protein (5 μg) that had been prepared as aforementioned was mixed with loading buffer and boiled at 95°C for 10 min. The proteins were then separated on a 12% SDS-PAGE gel. The separated proteins were transferred to polyvinylidene fluoride membranes (Merck KGaA, Darmstadt, Germany) for 30 min at 15 V. Following blocking in TBS-Tween-20 (0.1%) buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with an anti-PCNA antibody (1:20,000; cat. no. 13110; Cell Signaling Technology, Inc.) at 4°C overnight. The membranes were subsequently washed and incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (Ig)G antibody (1:4,000; cat. no. A10696; American Qualex, San Clemente, CA, USA) at room temperature for 1 h. The blots were washed and visualized with SuperSignal West Dura Extended Duration substrate (Thermo Fisher Scientific, Inc.). The bands were analyzed with the myECL Image software v3.0 (Thermo Fisher Scientific, Inc.). The membranes were subsequently stripped by Restore Western Blot Stripping buffer (Thermo Fisher Scientific, Inc.), and the same membranes were re-probed with an anti-β-actin antibody (1:5,000 dilution; cat. no. sc-47778; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight, which served as the protein loading control. The relative quantities of PCNA over β-actin were used to evaluate PCNA expression under different conditions. All western blot analyses were performed as three independent experiments.

**Cell cycle analysis by flow cytometry.** To analyze cell cycle distribution, DNA was stained with propidium iodide (PI; Nacalai Tesque, Inc., Kyoto, Japan). Briefly, DLD-1 cells were plated at a density of 2x10⁶ cells/100-mm dish in culture medium. The next day, the culture medium was replaced with 1% (v/v) extra light grade maple syrup (Extra), dark grade maple syrup (Dark) or without syrup (control). Following 72 h of incubation at 37°C, the cells were washed with PBS and fixed in ice-cold 70% ethanol at 4°C for 2 h. The cells were subsequently treated with 0.25 mg/ml RNase in PBS for 60 min at 37°C, followed by staining with 50 μg/ml PI in PBS for 30 min at 4°C in the dark. Cell proportion in different phases of the cell cycle was determined using a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FACS DIVA software v8.0.1 (BD Biosciences).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) using TaqMan array analysis.** Total RNA was extracted from treated DLD-1 cells using the GenElute Mammalian Total RNA Miniprep kit (cat. no. RTN70-1KT; Sigma-Aldrich; Merck KGaA), according to the manufacturer’s protocols. From the extracted RNA, cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (cat. no. 4368814; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocols. Analysis was performed using TaqMan Array Human Cyclins & Cell Cycle Regulation 96-Well Plates (cat. no. 4414123; Thermo Fisher Scientific, Inc.) in the 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocols. The thermocycling conditions were as follows: Denaturation at 95°C for 20 sec, followed by 40 cycles of amplification at 95°C for 3 sec and 60°C for 30 sec. The relative gene expression was calculated using the 2^{-ΔΔCq} method (39-43). The ΔΔCq method uses the normalized ΔCq value of each sample, which was calculated with 18S rRNA as the endogenous control gene. The ΔΔCq value is the difference between treated and control samples. Finally, the fold-change was determined as 2^{-ΔΔCq}.

**Statistical analysis.** All experiments were repeated at a minimum of three times. All data are presented as the mean ± standard error of the mean (SEM). The data were analyzed by one-way analysis of variance followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference. Computations were performed using GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA, USA).

**Results**

**Identification and semi-quantitative comparisons of differentially expressed proteins in maple syrup-treated DLD-1 cells.** To investigate the inhibitory effects of maple syrup on CRC proliferation, shotgun proteomics was used to examine the molecular profile of proteins that were regulated by maple syrup treatment. Applying the aforementioned search parameters, a total of 575 proteins were identified in DLD-1 cells treated with extra light grade maple syrup, and 549 proteins in DLD-1 cells treated with dark-colored maple syrup. Proteins were categorized into the ‘Extra’ and ‘Dark’ groups accordingly.

The proteins expressed in maple syrup-treated DLD-1 cells were further evaluated using a label-free semi-quantitative method based on spectral counting. The Rsc values were calculated for the proteins identified in the Extra and Dark groups. A positive Rsc value indicated increased expression with dark-colored maple syrup treatment, and a negative value indicated reduced expression with dark-colored maple syrup treatment (Fig. 1; light grey area). The NSAF value was also calculated for each protein identified in the Extra and Dark groups. Proteins with a >0.585 and <0.585 Rsc value were considered candidate dark-colored maple syrup-regulated proteins.

This semi-quantitative procedure resulted in the identification of 388 proteins that were differentially expressed with dark-colored maple syrup treatment (data not shown). Maple syrup treatment did not alter the expression of housekeeping proteins, including glyceraldehyde-3-phosphate dehydrogenase and histone H4 (Fig. 1).

**Functional annotation of proteins regulated by maple syrup.** GO analysis of the candidate dark-colored maple
syrup-regulated proteins was performed. GO terms associated with ‘pathway’ were searched for in DAVID (Fig. 2), and the focus was on proteins classified as associated with the ‘cell cycle’ (Table I).

**Effects of maple syrup on PCNA expression in DLD-1 cells.**
The expression of PCNA protein in maple syrup-treated DLD-1 cells was examined. The results of the present study indicated a significant decrease in PCNA expression with dark-colored maple syrup treatment (Dark) compared with that in the Extra group and the untreated (control) cells (Fig. 3).

**Effects of maple syrup on cell cycle progression of CRC cells.**
The present study also investigated whether the dark-colored maple syrup-induced decrease in PCNA expression affected cell cycle progression in DLD-1 cells. Flow cytometry analysis revealed a significantly increased cell population in the S phase (P<0.01), and a decreased population in the G2/M phase (P<0.05) in the Dark group compared with that in the Extra group and the control group (Fig. 4A and B).

**Effects of maple syrup on the expression of cell cycle-associated factors in CRC cells.**
Using the TaqMan Array Human Cyclins & Cell Cycle Regulation 96-Well Plate, qPCR was conducted to examine the molecular profile of cell cycle-associated mRNAs that were regulated by maple syrup treatment. RT-qPCR analysis was performed with DLD-1 cells treated with extra light or dark-colored maple syrup. In the Dark group, fold-changes were induced in the relative expression of cell cycle-associated factors. Expression levels in the Extra group were set as 1, and the fold-changes following dark-colored maple syrup treatment were evaluated using the DDCq method. Among the 44 tested cell cycle-associated genes, 12 genes indicated changes in expression of >2-fold in DLD-1 cells treated with dark-colored maple syrup treatment compared with extra light grade maple syrup (Fig. 5). It was indicated that dark-colored maple syrup treatment reduced cyclin-dependent kinase 4 (CDK4), CDK6 and transforming growth factor β1 (TGFB1) expression, and induced cyclin-dependent kinase inhibitor 2B (CDKN2B) expression (Fig. 5).

**Discussion**
In the present study, a gel-free LC-MS/MS-based proteomics approach was applied to examine the underlying mechanism of CRC cell growth inhibition by dark-colored maple syrup.
Using a semi-quantitative method of spectral counting, a total of 388 proteins were identified that indicated >1.5-fold changes in expression following maple syrup treatment. The roles of these identified proteins were examined with GO analysis, focusing on the functions of proteins classified as associated with the 'cell cycle', since these proteins serve important roles in the proliferation system. The study also focused on PCNA, which is a member of this pathway. A western blot analysis was subsequently performed to validate the spectral counting results, and to determine whether dark-colored maple syrup treatment led to decreased PCNA expression in DLD-1 cells.

PCNA has been reported to exhibit different behaviors depending on the cell cycle phase, and serves important roles in DNA replication and DNA repair (44-47). PCNA also recruits chromatin remodeling and epigenetic modification factors (48). During the S phase of the cell cycle, PCNA is reportedly localized in the active replication site, and has the ability to differentiate between early, mid and late...
S phase (49,50). Therefore, PCNA expression is considered to be associated with S-phase cell cycle progression.

The present study observed that dark-colored maple syrup treatment decreased PCNA expression, which induced the S-phase cell cycle arrest of DLD-1 cells. Previous reports also indicate that induction of S-phase cell cycle arrest is accompanied by decreased PCNA expression in a number of tumor cells, including in CRC cells treated with functional compounds, such as resveratrol and o xoaporphine metal complexes (51,52). Previous data also indicate that the induction of S-phase cell cycle arrest is associated with changes in the expression of cyclins and CDKs in these cells. Therefore, maple syrup treatment may influence the cell cycle-associated gene expression in CRC cells. Accordingly, we hypothesized that dark-colored maple syrup induced S-phase cell cycle arrest in DLD-1 cells, which may affect the expression of cell cycle-associated genes. The study findings supported this hypothesis.

CDK4/6 is a key kinase in cell cycle promotion, and is currently considered a molecular target for anticancer drug development (53,54). The CDK4/6 inhibitor palbociclib is clinically used for the treatment of advanced breast cancer (55,56). The results of the present study support the possibility that the bioactive compounds in dark-colored maple syrup may be useful in the development of novel anticancer drugs for treatment of CRC and other types of advanced cancer. In addition, CDKN2B is also known as a cell-cycle regulator via its interaction with CDK4 and CDK6, and tumor suppressor genes, including p53 and p18 (57). Therefore, it can be hypothesized that the bioactive compounds, which have been indicated to upregulate CDKN2B expression may be present in dark grade maple syrup and may be a useful resource in developing novel anticancer drugs. In addition, these bioactive compounds may have an inhibitory effect on the TGF signaling pathway by suppressing TGF-β1 expression by upregulating CDKN2B expression, since previous reports demonstrated that downregulation of CDKN2B expression induced an increase of TGFβ1 expression in human smooth muscle cells and umbilical vein endothelial cells (58). A previous study reported that the ginnalins-polyphenols that are present in maple syrup inhibit proliferation through S-phase cell cycle arrest (59). However, the effects of ginnalins on PCNA, CDK4/6 and CDKN2B expression in CRC cells are not well understood. Further studies are required to identify the bioactive compounds in dark-colored maple syrup that are responsible for inhibiting the expression of PCNA, CDK4/6 and/or CDKN2B, and therefore, inducing cell cycle arrest. Further clarification is required in order to examine whether dark-colored maple syrup inhibits proliferation through S-phase cell cycle arrest by regulating cell cycle-associated gene expression in other types of cancer cells.

In conclusion, the present study indicated that dark-colored maple syrup induced S-phase cell cycle arrest in CRC cells by reducing PCNA expression and regulating cell cycle-associated genes. These findings suggest that dark-colored maple syrup may be a useful dietary factor, with a potential preventative effect against CRC. Compounds in dark-colored maple syrup may also be useful for the development of novel anticancer drugs for colorectal cancer treatment.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
TY and AT designed the study and analyzed the data. TY and TN performed the experiments. TY drafted the manuscript. AT critically evaluated the study and the final version of the manuscript. All authors participated in discussion of the work and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

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Not applicable.

Competing interests
The authors declare that they have no competing interests.

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