Polyphenol-rich Muscadine Grape Extract Reduces Triple Negative Breast Cancer Metastasis in Mice with Changes in the Gut Microbiome

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Abstract

Background

Triple negative breast cancer (TNBC) has a high propensity to metastasize and no treatments are available to slow or prevent metastatic progression. The goal of this study is to determine whether a proprietary high-polyphenol content muscadine grape extract (MGE) inhibits TNBC metastasis.

Methods

4T1 TNBC cells were injected into the mammary fat pad of 6-week-old female Balb/c mice. After 2 weeks, tumors were surgically removed and mice were placed into a control (n=8) or treatment group that received 0.1 mg/mL total phenolics MGE in the drinking water (n=8) for 4 weeks. Immunohistochemistry (Ki67, α-SMA) and hemotoxylin and eosin staining were used to quantify metastases. Gut microbial composition was determined by 16S rRNA sequencing and short chain fatty acids (SCFAs) were detected by gas chromatography. MDA-MB-231, BT-549 and 4T1 TNBC cell motility and cytoskeletal organization was assessed *in vitro* by scratch wound migration and confocal microscopy, respectively. Data were evaluated by student’s *t*-test.

Results

MGE reduced metastatic proliferation in mouse lungs (33.3%) and livers (58.3%) and decreased the number (51.1%) and size (17.4%) of liver metastases, resulting in a 55.7% reduction in metastatic tumor burden (*P* < 0.01). Serum IL-6 was reduced 99.6% in MGE-treated mice (*P* = 0.06). MGE attenuated migration, altered cytoskeletal organization, and reduced RHAMM expression in TNBC cells (*P* < 0.05). The gut microbiota, a mediator of polyphenolic bioactivities, was altered significantly in MGE-treated mice; MGE increased the alpha diversity (7.14%), Firmicutes/Bacteroidetes ratio (2-fold), relative abundance of butyrate-producing genera, and butyrate (3-fold) (*P* < 0.05). Butyrate inhibited 4T1 cell proliferation and migration, suggesting butyrate contributes to MGE’s anti-metastatic activities (*P* < 0.05).

Conclusion

Our results indicate that MGE may be an effective adjuvant therapy to reduce TNBC metastatic progression.
Key words: triple negative breast cancer, nutraceutical, gut microbiota, migration, proliferation, butyrate, metastasis
Background

Metastasis accounts for 90% of cancer-related deaths and is a common characteristic of patients with triple negative breast cancer (TNBC). TNBC, an aggressive subtype of breast cancer, accounts for 12-17% of breast cancer cases. TNBC is characterized by a lack of estrogen and progesterone receptors as well as overexpression of the human epidermal growth factor receptor type 2 (HER2) [1, 2]; no targeted therapies are currently available due to this lack of receptor expression. Patients with TNBC are four times more likely to develop visceral metastasis within 5 years of diagnosis compared to other breast cancer subtypes [3] and about 34% of TNBC patients experienced distant recurrence in an average of 2.6 years after diagnosis [4].

Metastatic development is a multifaceted process involving many interdependent steps [5]. In the metastatic cascade, cancer cells invade the surrounding stroma, intravasate into the circulatory system, and extravasate to a distant site [6, 7]. Because metastasis is multifaceted, a therapeutic such as a complex plant extract, with multiple bioactivities, may serve as an effective treatment option. Muscadine grapes contain high amounts of polyphenols, such as gallic acid, ellagic acid, catechins, and cyanidins [8], which exhibit anti-oxidant, anti-microbial and anti-cancer properties [9]. Muscadine grape extracts (MGEs) or their individual components reduced proliferation and/or migration of colon, prostate and breast cancer cells [10-19]. We previously showed that a proprietary MGE effectively reduced TNBC cell proliferation in vitro and tumor growth in vivo at clinically relevant concentrations [20].

Polyphenols modulate gut microbiota composition to confer physiological effects [21, 22]. Liu et al. [23] showed that the gut microbiome was a critical mediator of the anti-inflammatory activities of a grape seed proanthocyanidin extract (GSPE) in high fat diet-fed C57BL/6 mice. Han et al. [24] found that a grape extract increased the Firmicutes to Bacteroidetes ratio (F/B) and alpha diversity in high-fat high-fructose fed C57BL/6 mice in conjunction with partially restored gut dysbiosis. Likewise, Sheng et al. [25] showed that a GSPE restored microbiota homeostasis in colitis-induced C57BL/6 mice by reducing Bacteroidetes, Dubosiella, and Veillonella, and increasing Verrucomicrobia, Akkermansia, and the F/B ratio [25]. Gut dysbiosis is implicated in a variety of diseases, including breast cancer [26]; breast cancer patients have reduced alpha diversity compared to their healthy counterparts [27]. Recently, Buchta Rosean et al. [28] showed that gut microbiome dysbiosis promotes metastatic dissemination in a mouse model of hormone-positive breast cancer. In addition, depletion of the gut microbiome by
antibiotic treatment significantly reduced pancreatic, colon, and melanoma liver metastases, suggesting the importance of the gut microbiome in metastatic progression [29].

The goal of this study was to determine whether a proprietary MGE decreases TNBC metastasis in vivo and if changes in the gut microbiome are associated with the reduction in tumor burden.

Methods

MGE

A proprietary MGE derived from the seeds and skin of muscadine grapes of the Carlos variety was purchased from Piedmont Research and Development Corp. as previously described [20, 30]. MGE concentrations are reported as the amount of total phenolics quantified using the Folin-Ciocalteu reagent, with gallic acid as the standard. Unless otherwise noted, 20 μg total phenolics/mL of MGE was used in in vitro experiments.

Animal Model

The Wake Forest School of Medicine Animal Care and Use Committee approved the animal experiment. The 4th inguinal mammary fat pads of 6-week old female Balb/c mice (Harlan) were injected with $2.5 \times 10^5$ actively growing mouse 4T1 TNBC cells suspended in 100 μL phosphate-buffered saline (PBS). The mice were group housed in cages with HEPA-filtered air on 12-h light/dark cycles and were fed standard mouse chow ad libitum. Two weeks post cell injection, the tumors were removed surgically and mice were randomized into a control group receiving no treatment or the MGE group receiving 0.1 mg total phenolics/mL of MGE in the drinking water (corresponding to a dose of 0.5 mg total phenolics/mouse/day for a 25 g mouse). Four weeks after the surgery, mice were euthanized; blood and fecal samples were collected.

Immunohistochemistry

Whole lungs and the left lateral lobe of livers were fixed in 4% paraformaldehyde and immersed in 70% ethanol prior to paraffin embedding and 5 μm sectioning. Staining for Ki67 (1:100; RM-9106-S; Thermo Fisher) and α-smooth muscle actin (α-SMA;1:100; ab32575; Abcam) was performed using the Opal™ Multiplex IHC kit and fluorescent staining protocol with 4′,6-diamidino-2-phenylindole (DAPI) as a counterstain (Perkin-Elmer).
Antibody verification information is found in Supplemental Table 1. Negative controls with only the secondary antibody were included to account for non-specific binding. Cell positivity was determined using inForm® software (Perkin-Elmer).

Liver Metastasis Quantification

For each animal, five separate tissue sections from the formalin-fixed livers at least 50 µm apart were stained with hematoxylin and eosin (Newcomer Supply). Images of stained tissues were collected from four separate quadrants of each liver section using the Quantitative Pathology Imaging System Mantra™ microscope (Perkin-Elmer). Liver metastases were analyzed using inForm® 2.2 by training the software to detect healthy tissue, metastases, and background. Vessels detected as metastases by the software were manually removed from the analysis.

Serum IL-6 Analysis

IL-6 was quantified in mouse serum samples using an ELISA kit (ab100712) according to the manufacturer’s protocol (Abcam).

Cell Culture

4T1 (CRL-2539) mouse stage IV breast cancer cells derived from Balb/cfC3H mice, MDA-MB-231 (HTB-26) human mammary adenocarcinoma cells and BT-549 (HTB-122) ductal carcinoma cells were obtained from the American Tissue Culture Collection. All cell lines were authenticated by IDEXX BioAnalytics using short tandem repeat (STR) analysis within 6 months of the completion of experiments. 4T1 and BT-549 cells were grown in Gibco® RPMI-1640 medium and MDA-MB-231 cells were grown in Gibco® DMEM at 37°C (Thermo Fisher Scientific). All media was supplemented with 100 µg/mL penicillin, 100 units/mL streptomycin, 15 mM HEPES, 2 mM L-glutamine and 10% fetal bovine serum (FBS).

Cell Proliferation

Sodium butyrate-treated cells transfected with the IncuCyte® NucLight™ Red Lentivirus Reagent (EF1α, Puro) were monitored for proliferation with the IncuCyte® ZOOM System according to the manufacturer’s protocol (Sartorius).
Cell Migration

The IncuCyte® Scratch Wound Cell Migration system was used to quantify cell migration according to the manufacturer’s protocol. Briefly, cell migration was measured by phase contrast mask with the final wound width subtracted from the initial wound width to obtain the distance migrated. Cell migration was analyzed after 12 h of MGE or 18 h of sodium butyrate treatment. To ensure the observed effects on cell migration were measured before significant proliferation occurred, simple proliferation of IncuCyte® NucLight™ Red-transfected cells was monitored during the scratch wound migration assay by recording the change in nuclei count throughout the migration experiment (Supplementary Fig. 2). MGE effects on migration were assessed within timeframes when the fractional rate of change of proliferation was less than 5%.

Confocal Microscopy

Cells were fixed with 3.7% formaldehyde (methanol-free) in PBS, permeabilized in 1% bovine serum albumin (BSA) with 0.1% Triton X-100 in PBS, and incubated with 1:40 phalloidin (A12379; Thermo Fisher) and 1:1000 DAPI (Perkin-Elmer) in 1% BSA-PBS. Slides were mounted with Prolong Diamond and 24x50x1.5 coverslips (Thermo Fisher). Images were taken with the Zeiss LSM 880 confocal microscope with the Plan-Apo 40x/0.95 and Plan-Apo63x/1.4 oil objectives and lasers set to 405 nm and 488 nm at the Wake Forest University Microscopy Core Facility. Images were quantified using the CellProfiler software [31].

RNA Isolation and qRT-PCR

RNA was isolated using the Trizol® reagent according to the manufacturer’s protocol (Thermo Fisher Scientific, Waltham, MA). Total RNA (1 μg) was reverse transcribed; the resultant cDNA was added to TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with gene-specific primer/probe sets and amplified. All reactions were performed in triplicate; 18S ribosomal RNA amplified using the TaqMan rRNA control kit (Applied Biosystems) served as an internal control. Relative gene expression was quantified using the $2^{-ΔΔCt}$ method.

Western Blot
Cell lysates were prepared in Triton lysis buffer (32) and protein concentration was determined using the Bio-Rad Bradford protein assay (Hercules, CA) with BSA as a standard. Western blots were performed using Bio-Rad reagents and equipment (Hercules, CA). 10% Mini-PROTEAN® TGX™ Precast Protein Gels were loaded with 20 µg protein/well and transferred onto Immun-Blot® PVDF membranes. Anti-CD168/RHAMM (1:1000, ab124729; Abcam; Cambridge, UK) was diluted in 5% Blotting-Grade Blocker in Tris-buffered saline (TBS) with 0.1% Tween® and applied to membranes overnight at 4°C with gentle agitation. Antibody verification information is found in Supplemental Table 1. Western blots were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA) and imaged using the ChemiDoc™ Touch Imaging System. Image Lab™ Software was used for analysis of immunoreactive bands, which were normalized to total protein/lane using a stain free blot.

Microbiome Analysis

Fecal samples were collected from the distal colon at the time of sacrifice and flash frozen to -80°C. 16S rRNA analysis and quantification determined the relative abundance of bacteria based on amplicon counts of the V4 region (Microbiome Insights). Alpha diversity was determined by Shannon index on raw operational taxonomic units (OTU) abundance tables after filtering out contaminants [32, 33].

Short Chain Fatty Acid Quantification

Short chain fatty acids (SCFA) were extracted from fecal samples collected from the distal colon of mice at the time of sacrifice using the method previously described [34]. SCFAs were detected by gas chromatography (Trace 1310 Gas Chromatograph) using the Thermo TG-WAXMS A GC column (30 m, 0.32 mm, 0.25 µm) coupled to a flame ionization detector (Thermo Fisher) by Microbiome Insights.

Statistical Analysis

Statistical analysis was performed by student’s t-test or one-way ANOVA using the GraphPad Prism 6 software. Results were considered significant when $P<0.05$. All data are presented as mean ± SEM.

Results
MGE reduces metastatic triple negative breast cancer in vivo

In our clinically relevant 4T1 metastatic mouse model (Supplemental Fig. 1), metastases were present in mouse lungs and livers, as previously observed [35]. MGE had no effect on mouse weight (22.1 ± 0.7 vs 21.0 ± 0.4 g, \(P = 0.19\)). Due to the aggressiveness of the model, lung metastases were too widespread to count and measure. Metastatic lung cell proliferation was quantified as Ki67+/α-SMA- cells to exclude proliferating fibroblasts. Fig. 1a shows representative images of mouse lung tissues immunostained with Ki67 (green) and/or α-SMA (orange) with DAPI (blue) as a nuclear counterstain. Ki67 positivity within the lungs was reduced significantly in MGE-treated mice compared to the control group (9.3 ± 0.9% vs 6.2 ± 0.7% positive cells, \(P < 0.01\); Fig. 1b). MGE also decreased Ki67 positivity within the liver (1.8 ± 0.4% vs 0.75 ± 0.1% positive cells, \(P < 0.05\); Fig. 1c, d). The reduction in Ki67 positivity suggests that MGE reduces proliferation of metastatic 4T1 cancer cells in the lung and liver.

MGE decreased the number (4.3 ± 0.65 vs 2.1 ± 0.42 metastases/field, \(P < 0.01\)) (Fig. 2a) and size (1335 ± 49 vs 1043 ± 47 pixels, \(P < 0.001\)) (Fig. 2b) of individual mouse liver metastases. Together, these reductions led to a 55% decrease in total liver metastasis area per field in mice treated with MGE compared to control mice, indicating reduced tumor burden (5758 ± 1168 vs 2564 ± 628 pixels, \(P < 0.05\); Fig. 2c, d). Metastases were quantified using an automated analysis mask trained to detect healthy liver, metastases, or background from hematoxylin and eosin stained liver tissues. Serum IL-6 was quantified because increased concentrations of IL-6 are associated with breast cancer liver metastases in humans [36] and a grape powder reduced serum IL-6 in an inflammatory mouse model [37]. Serum IL-6 was negligible in MGE-treated mice compared to serum from control mice (53.6 ± 24 vs 0.23 ± 0.23 pg/mL, \(P = 0.06\); Fig. 2e). Taken together, these results show that MGE reduces TNBC metastasis in vivo.

MGE reduces triple negative breast cancer cell migration and alters cytoskeletal arrangement in vitro

The effect of MGE on cell migration was assessed in vitro, as cell motility plays a critical role in the metastatic cascade. Confluent monolayers of TNBC cells were scratched to create a denuded area and cell migration was measured in MDA-MB-231 (Fig. 3a), BT-549 (Fig. 3c), and 4T1 (Fig. 3e) cells. MGE inhibited migration of MDA-MB-231 cells by 29.3% (416 ± 45 μm vs. 294 ± 17 μm, \(P < 0.05\)), BT-549 cells by 44.0% (193 ± 25 μm vs. 133 ± 19 μm, \(P < 0.01\)), and 4T1 cells by 38.0% (78 ± 10 μm vs. 48 ± 6 μm, \(P < 0.01\)).
108 ± 16 μm, P < 0.05) and 4T1 cells by 59.6% (203 ± 30.9 vs 82.1 ± 29.5 μm, P < 0.01) (Fig. 3b, d, f). The reduction in migration suggests that MGE reduces TNBC cell motility.

The effect of MGE on TNBC cell structure was assessed since cytoskeletal reorganization is crucial for cell migration. MGE altered the shape of MDA-MB-231 cells compared to control cells. MGE increased cell area by 49%, as quantified by an increase in both cell length and width (P < 0.05; Fig. 4a, b, c), and increased cell eccentricity, indicating that MGE-treated cells are less circular than untreated cells (P < 0.001; Fig. 4d). Representative images also show cell polarization in MDA-MB-231 cells (marked by the asterisk in Fig. 4e); in contrast, MGE-treated cells lack directionality or polarity.

Cell size was reduced by 60% in MGE-treated BT-549 cells compared to control cells, which correlated with reduced cell length and width (P < 0.0001; Fig. 5a, b, c). MGE had no effect on BT-549 cell eccentricity (Fig. 5d). As shown in Fig. 5e, MGE-treated BT-549 cells displayed marked morphological differences compared to control cells; untreated BT-549 cells are spindle shaped with outstretched filopodia, which play important roles in cell migration, while MGE-treated BT-549 cells display few to no filopodia [38].

4T1 cells treated with MGE also show marked differences in actin arrangement compared to control cells (Fig. 6). However, variations in cell size could not be accurately quantified as 4T1 cells grow in aggregates. As indicated by the white arrows in Panel A, untreated 4T1 cells contained parallel f-actin fibers, indicative of stress fibers [39]. In contrast, MGE-treated cells displayed a lack of stress fibers, punctuate f-actin staining (dotted arrow in Panel B), and an increase in f-actin staining around the cell perimeter (solid arrow in Panel B). Since cell structure is a critical component of cell migration, the MGE-induced alterations in TNBC cytoskeletal organization may contribute to the effects of MGE on cell migration and consequently participate in anti-metastatic actions of MGE in vivo.

MGE inhibits metastasis protein RHAMM in vitro

Receptor for hyaluronan-mediated motility (RHAMM), a prominent metastasis related protein that can associate with microtubules and actin filaments to promote cell motility, is implicated in breast cancer cell migration [40, 41]. HMMR mRNA was reduced by 75%, 94%, and 78% in MDA-MB-231 (Fig. 7a), BT-549 (Fig. 7c) and 4T1 (Fig. 7e) cells treated with MGE for 24 h, respectively, compared to control cells (P < 0.01). RHAMM was decreased by 84%, 81%, and 55% in MDA-MB-231 (Fig. 7b), BT-549 (Fig. 7d) and 4T1 (Fig. 7f) MGE-treated cells,
respectively, compared to untreated cells \( (P < 0.001) \). Because RHAMM is involved in cell migration and metastasis, it may represent a critical target of MGE [40] [42].

MGE alters the gut microbiome in a triple negative breast cancer metastatic mouse model

Polyphenols are metabolized by the gut microbiota to generate products, which also have important biological activities [43, 44]. Mice treated with MGE showed a significant increase in fecal alpha diversity \( (4.2 \pm 0.1 \text{ vs } 4.5 \pm 0.1 \text{ Shannon index}, P < 0.05; \text{Fig. 8a}) \). At the phylum level, MGE reduced the relative abundance of Bacteroidetes \( (0.73 \pm 0.04 \text{ vs } 0.57 \pm 0.04, P < 0.05; \text{Fig. 8b}) \) and increased the relative abundance of Firmicutes \( (0.25 \pm 0.04 \text{ vs } 0.40 \pm 0.03, P < 0.05; \text{Fig. 8c}) \), increasing the F/B ratio \( (0.38 \pm 0.07 \text{ vs } 0.76 \pm 0.12, P < 0.05; \text{Fig. 8d}) \) compared to untreated mice, which suggests that MGE increases gut microbiota energy harvest [45].

MGE increased the relative abundance of several genera belonging to the Firmicutes phylum. The feces of MGE-treated mice had a 2-fold increase in Clostridium \( (P < 0.05) \), a 5-fold increase in Ruminococcus \( (P < 0.05) \), and a 2.5-fold increase in Butyricicoccus \( (P < 0.05) \) genera compared to the feces of control mice (Fig. 8e, g, h).

MGE also increased the relative abundance of bacteria belonging to unclassified genera from the Lachnospiraceae and Lactobacillaceae families by 2- and 7.5-fold, respectively \( (P < 0.05) \) (Fig. 8f, i). Among these genera, the Clostridium, Butyricicoccus, Ruminococcus and unclassified Lachnospiraceae genera all produce butyrate, a SCFA with anti-inflammatory properties [46].

MGE shifts short chain fatty acid composition in the gut towards increased butyrate

SCFAs are microbial metabolites that elicit biological effects in the host. MGE significantly reduced acetate \( (9.4 \pm 1.6 \text{ vs } 2.0 \pm 1.4 \text{ mmol SCFA/kg feces, } P < 0.01) \) and propionate \( (7.5 \pm 1.0 \text{ vs } 0.0 \pm 0.0 \text{ mmol SCFA/kg feces, } P < 0.0001) \) within mouse fecal samples compared to control mice, indicating lower acetate and propionate in the gut (Fig. 9a, b). While there was no difference in the absolute amount of butyrate between the control and MGE-treated groups (Fig. 9c), the extract shifted the SCFA composition to favor butyrate by increasing the relative abundance of butyrate compared to all other SCFAs \( (25.0 \pm 2.7\% \text{ vs } 75.3 \pm 15.5\%, P < 0.01) \) (Fig. 9d).

Butyrate reduced 4T1 cell proliferation and migration in a dose- and time-dependent manner \( (P < 0.05; \text{Fig. 9e, f, g}) \). Treatment with 1 mM of butyrate, a concentration below the butyrate levels measured in mouse fecal samples, reduced cell migration by 12.5\% after 18 h \( (P < 0.05) \), a time point with minimal changes in cell proliferation.
These results show that, at physiologically relevant concentrations, butyrate inhibited 4T1 TNBC cell proliferation and migration, which could contribute to the MGE-induced metastasis reduction in vivo.

Discussion

Our results show that MGE reduced TNBC metastasis in a clinically relevant TNBC metastatic mouse model, decreased TNBC cell migration in vitro, altered cytoskeletal arrangement, and reduced metastasis protein RHAMM. Significant changes in gut microbiota composition were also associated with the anti-metastatic activities of the extract. Further, MGE increased the relative abundance of butyrate and the fatty acid inhibited 4T1 cell proliferation and migration, indicating that a microbial byproduct may contribute to the anti-metastatic effects of MGE.

MGE reduced the number and size of individual tumor metastases, suggesting that the extract may block the metastatic cascade and inhibit proliferation of metastatic tumors. Repeated cycles of cell protrusion, adhesion, contraction and retraction participate in the migration of most cancer cells, involving repeated reorganization of the actin cytoskeleton [47, 48]. The different effects of MGE on cell cytoskeletal organization in individual TNBC cell lines may result from the heterogeneous genetic backgrounds of TNBC cells, similar to TNBC heterogeneity among patients. Reductions in cell polarity, filopodia, stress fibers, or peripheral f-actin staining, which are all necessary at the leading edge of migrating cells, could each contribute to a decrease in TNBC cell migration [48-50]. The MGE-mediated differential effects on cytoskeletal organization may be regulated by the Rho family GTPase Cdc42, which is a master regulator of cell polarity, or by the WAVE/WASP and Arp2/3 pathway, which participates in actin-mediated protrusions to confer changes in cell morphology [51, 52]. In addition, punctuated actin staining may result from effects of MGE on actin disassembly [53]. The precise molecular mechanism for the alterations in cytoskeletal organization by MGE is currently under investigation.

MGE reduced RHAMM and HMMR, the gene encoding RHAMM, in all three TNBC cell lines. RHAMM is a multi-functional protein found on the cell surface, in the cytoplasm, or in the nucleus and participates in cell motility regulation [41]. RHAMM overexpression in primary breast tumors is associated with a poor prognosis and lymph node metastases, suggesting that RHAMM has an important role in breast cancer metastasis [42]. RHAMM can associate with ERK1/2, Src, and FAK to promote cell migration [40, 54-59] and loss of RHAMM reduces focal
adhesion turnover, filopodia, and cell locomotion [40, 58, 59]. Therefore, the reduction of RHAMM by MGE in TNBC cells could lead to the reduction in cell migration and metastasis.

MGE reduced Ki67 in the lungs and livers of mice with TNBC metastasis in association with effects on the gut microbiota. Polyphenols alter the gut microbiome and are metabolized by the microbiota into distinct bioactive compounds [21, 60, 61]. MGE increased alpha diversity, indicating that the extract improved gut health by increasing robustness and functional capability [62, 63]. Likewise, MGE increased the F/B ratio, which improves the health of the mice by increasing the ability of the gut microbiota to extract energy from the diet to reduce weight loss and increase overall fitness [45, 64-66].

MGE also increased the relative abundance of butyric acid in fecal samples compared to control mice, which may be more beneficial than changes in total SCFA [67-70]. MGE significantly reduced acetic and propionic acid, which are the main products of the Bacteroidetes phylum [71]. The reduction in acetic and propionic acid resulted in an increase in the relative abundance of butyric acid. Acetate, propionate, and butyrate associate with the same receptors on the apical membrane of colonocytes, including monocarboxylate transporters and the G-protein coupled receptors (GPR) GPR41/Ffar3 and GPR43/Ffar2. An increase in the relative abundance of butyrate would increase the proportion of butyrate available to the transporters and receptors [72, 73]. Consequently, in MGE-treated mice, butyrate may outcompete acetate and propionate for these receptors to cause a greater biological impact.

Compared to acetate and propionate, butyrate has the most potent anti-inflammatory effects and thus may contribute to the reduction in serum IL-6 in MGE-treated mice [74]. Butyrate modulates gene expression through inhibition of histone deacetylases and reduces cancer cell proliferation and migration [75-77]. Since butyrate can travel through the portal vein to have direct contact with the liver, MGE-induced increases in butyrate may contribute to the reduction in liver metastatic tumor burden [78]. Furthermore, butyrate concentrations in the blood can range from 0.1-1 mM, indicating that butyrate in the lung and liver may be present at concentrations that would inhibit 4T1 cell migration and metastatic proliferation [79].

Conclusions
For the first time, we showed that MGE inhibits TNBC metastasis in mice concomitant with MGE-induced changes in TNBC cell morphology and migration as well as the gut microbiota, which may mediate anti-cancer effects of MGE. In an ongoing Phase I clinical trial in patients with solid tumors, MGE has a favorable safety profile suggesting that the extract can be administered to patients at concentrations similar to those used in our studies [80]. Since TNBC patients currently have no therapeutic options to reduce metastatic progression after standard-of-care, MGE may be an effective adjuvant to reduce TNBC metastases.

Abbreviations: MGE, muscadine grape extract; TNBC, triple negative breast cancer; GSPE, grape seed proanthocyanidin extract; PBS, phosphate buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; α-SMA, alpha smooth muscle actin; F/B, Firmicutes to Bacteroidetes ratio

Declarations

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Authors’ contributions: MC, EAT, and PEG designed research; MC and NNA conducted research; MC and BW analyzed data; H B-H assisted with confocal microscopy; MC, EAT, and PEG wrote the paper and had primary responsibility for final content. All authors read and approved the final paper.

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