

Grape antioxidant dietary fiber inhibits intestinal polyposis in *Apc*^{Min/+} mice: relation to cell cycle and immune response

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Epidemiological and experimental studies suggest that fiber and phenolic compounds might have a protective effect on the development of colon cancer in humans. Accordingly, we assessed the chemopreventive efficacy and associated mechanisms of action of a lyophilized red grape pomace containing proanthocyanidin (PA)-rich dietary fiber [grape antioxidant dietary fiber (GADF)] on spontaneous intestinal tumorigenesis in the *Apc*^{Min/+} mouse model. Mice were fed a standard diet (control group) or a 1% (w/w) GADF-supplemented diet (GADF group) for 6 weeks. GADF supplementation greatly reduced intestinal tumorigenesis, significantly decreasing the total number of polyps by 76%. Moreover, size distribution analysis showed a considerable reduction in all polyp size categories [diameter <1 mm (65%), 1–2 mm (67%) and >2 mm (87%)]. In terms of polyp formation in the proximal, middle and distal portions of the small intestine, a decrease of 76, 81 and 73% was observed, respectively. Putative molecular mechanisms underlying the inhibition of intestinal tumorigenesis were investigated by comparison of microarray expression profiles of GADF-treated and non-treated mice. We observed that the effects of GADF are mainly associated with the induction of a G₁ cell cycle arrest and the downregulation of genes related to the immune response and inflammation. Our findings show for the first time the efficacy and associated mechanisms of action of GADF against intestinal tumorigenesis in *Apc*^{Min/+} mice, suggesting its potential for the prevention of colorectal cancer.

Introduction

Most grape dietary fiber and phenolics accumulate in the fruit skins, seed and pulp, which after the manufacture of grape juice and wine remains as pomace. After production, this processed raw material becomes a by-product and is used as fertilizer, animal feed or disposed in dumps, being a great waste of health-promoting compounds.

Abbreviations: *Apc*, adenomatous polyposis coli; FC, fold change; GADF, grape antioxidant dietary fiber; KEGG, Kyoto Encyclopedia of Genes and Genomes; PA, proanthocyanidin.

As there is some evidence suggesting that dietary intake of vegetables and fruits, rich in fiber and phenolic compounds, is associated with a decrease in the risk of developing colorectal cancer (1), further study of these by-products may help to define their application as colon cancer chemopreventive agents.

Grape antioxidant dietary fiber (GADF), here in the form of lyophilized red grape pomace, is a wine processing by-product from red grape that is rich in dietary fiber and phenolics. It contains a large amount (13% w/w) of non-extractable polymeric PA, mainly (epi)catechin-based polymers that are part of the dietary fiber fraction together with lignins and polysaccharides. During its transit along the intestinal tract, the small soluble phenolics are absorbed and the remaining PA progressively release (epi)catechin units that are then absorbed and metabolized. The remaining polymeric PA is cleaved by the intestinal microbiota into smaller species such as phenolic acids, which in turn are absorbed and metabolized (2). Previous studies in male Wistar rats have shown that GADF reduces mucosal apoptosis, probably due to modulation of the glutathione redox system and endogenous antioxidant enzymes (3). Recently, Lizárraga *et al.* (4) reported that the inclusion of GADF in the mouse diet protects the normal colon tissue against polyp development through alterations in the expression of tumor suppressor genes and proto-oncogenes as well as the modulation of enzymes pertaining to the xenobiotic detoxifying system and endogenous antioxidant cell defenses. Together, these results suggest that GADF could be an effective chemopreventive agent against colorectal cancer. However, the efficacy of GADF as a chemopreventive agent needs to be established in well-defined preclinical models of colon cancer before embarking on clinical trials.

The *Apc*^{Min/+} mouse is a model of colon cancer that harbors a dominant germ-line mutation at codon 850 of the homolog of the human adenomatous polyposis coli (*Apc*) gene, which results in a defective protein product that predisposes the mice to spontaneously develop pre-neoplastic intestinal polyps (5). APC function is linked to the Wnt signaling pathway, in which it operates by activating β -catenin degradation. Therefore, mutation of the *Apc* gene produces cytosolic accumulation and an increase in the nuclear translocation of β -catenin. In the nucleus, β -catenin activates the transcription factor T cell factor/lymphoid enhancer factor, giving rise to an increase in the expression of genes regulating cell proliferation and predisposing the cells to the formation of tumors. Mutations in the *Apc* gene have been directly implicated in the development of both human familial adenomatous polyposis and sporadic colorectal cancer (6). Hence, the *Apc*^{Min/+} mouse model is considered an analog of human intestinal tumorigenesis and has been widely used to study the effects of dietary and pharmaceutical agents on human colon cancer prevention. Here, we assessed the efficacy and associated molecular mechanisms of action of GADF consumption on spontaneous intestinal tumorigenesis in *Apc*^{Min/+} mice.

Materials and methods

Grape antioxidant dietary fiber

GADF was obtained from red grapes (the *Cencibel* variety) harvested in the vintage year 2005 in *La Mancha* region in Spain, as described in the Spanish patents registered under the numbers 2259258 and 2130092. The percentage composition of GADF used in this work was as follows: dietary fiber, 73±0.8 (58±0.8 comprising an indigestible fraction of insoluble compounds such as lignin and PAs and 16±0.1 of a soluble fraction constituted by pectins and hemicellulose); polymeric PAs associated with insoluble dietary fiber, 15±0.2; fat, 8±0.5; protein, 11±0.5 and ash, 5±0.2. More than 100 phenolic compounds (not associated with dietary fiber) such as phenolic acids, anthocyanidins, catechins and other flavonoids have been detected in GADF (7).

Animals and diet

We used male *Apc*^{Min/+} mice aged 5 weeks from Jackson Laboratories (Bangor, ME). Animals were housed in plastic cages at 22°C and 50% humidity, with

a 12:12h light/dark cycle, according to European Union Regulations. The experimental protocols were approved by the experimental animal ethical research committee of the University of Barcelona in accordance with current regulations for the use and handling of experimental animals. After 7 days of acclimatization during which they received a standard diet (Teklad Global 18% Protein rodent diet), the animals were randomly divided into two groups, with 12 and 10 mice per group (control and GADF, respectively). Control mice continued to be fed the standard diet, and the GADF-treated group was fed a special diet comprising the basal diet (Teklad Global 18% Protein rodent diet) supplemented with GADF at 1% w/w, which mimicked the recommended dietary fiber intake for humans (8). Diets were purchased from Harlan Interfauna Iberica S.L. (Barcelona, Spain). Both food and water were supplied *ad libitum* throughout the experiment. Throughout the 6 week treatment period, mice were observed for any signs of toxicity, and body weight and food and water intake were recorded weekly. At the end of the 6 weeks, the animals were starved overnight and then anesthetized with volatile isoflurane (Esteve, Barcelona, Spain). Finally, animals were killed by an overdose of anesthesia.

Measurement of intestinal polyps

Apc^{Min/+} mice develop polyps in both the small and large intestine, although more intestinal adenomas are observed in the small intestine. Therefore, after killing, the small intestine was excised from each mouse. Immediately after killing, the small intestine was cut longitudinally and rinsed with phosphate-buffered saline solution (pH 7.4) to remove the intestinal contents. The intestines were pinned flat on cardboard and then fixed for 1 day in 4% neutral-buffered formalin solution (v/v; pH 7.4). Intestinal sections were stored at room temperature in 1% neutral-buffered formalin solution (v/v) until further analysis. In order to facilitate tumor quantification and identification, the small intestine was divided into three equal sections: proximal, medial and distal. Thereafter, the small intestine sections were stained in phosphate-buffered saline solution (pH 7.4) and 0.1% (v/v) methylene blue. Using a stereomicroscope and a measured grid, the number of tumors and their dimensions in each small intestine section were determined. The size of each tumor was categorized as <1, 1–1.9 or ≥2 mm.

RNA isolation and gene profiling by Affymetrix microarrays

Large intestine was removed and placed on a plastic plate, which was kept at 4°C on ice. After removal of the rectum, the colon was opened longitudinally with fine scissors, and mucus, feces and polyps were removed. The normal colonic mucosal layer was incubated in Trizol (Invitrogen, Carlsbad, CA) for 3 min and scraped off the muscle layer using the edge of a sterile glass slide. Cells were transferred into 800 µl Trizol, homogenized by pipetting and stored at –80°C until RNA isolation. Total RNA was isolated using a combination of the Trizol method (Invitrogen) and the RNeasy Mini kit and DNase I treatment (Qiagen, Germantown, MD) according to the manufacturer's protocols. RNA integrity was tested using lab-on-a-chip technology on the BioAnalyzer 2100 (Agilent, Palo Alto, CA) and only a RNA integrity number >8 was accepted. Affymetrix microarrays using the Mouse Genome 430 2.0 platforms were performed according to the protocols published by the manufacturer (Affymetrix). We analyzed five RNA samples chosen at random from each group, five for the control and five for GADF group.

Microarray data analyses

Data were standardized using the Robust Multi-array Average method (9) and quantile normalization. Differential gene expression was assessed using the *limma* (10) package from Bioconductor. Multiple testing adjustment of *P*-value was conducted according to Benjamini *et al.* (11). Biochemical pathway analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper. This is a collection of KEGG mapping tools for KEGG pathway mapping. The tool 'Search and Color Pathway' was used to overlay gene expression results from microarrays onto biochemical pathways found in the KEGG. Gene expression levels were denoted using color codes displayed along the pathway by gene symbol boxes. Different shapes and patterns were used to represent induced and suppressed gene expression. Enrichment analysis was based on MetaCore™, an integrated knowledge database and software suite for pathway analysis of experimental data and gene lists. Enrichment analysis consists of matching the gene IDs of possible targets for the 'common', 'similar' and 'unique' sets with gene IDs in functional ontologies in MetaCore. The probability of a random intersection between a set of IDs and the size of the target list with ontology entities is estimated by the *P*-value of the hypergeometric intersection. A lower *P*-value means higher relevance of the entity to the data set, which results in higher rating for the entity. Use of the false discovery rate (adjusted *P*-value) allows processes with doubtful significance for the current experiment to be rejected and ensures that the findings are not contaminated with false positives.

Reverse transcriptase–real-time PCR

One microgram of total RNA was reverse transcribed following the instructions of the manufacturer (Invitrogen). The complementary DNA product was

used for subsequent amplification by real-time PCR in an ABI Prism 7000 Sequence Detection System using gene-specific primers following the manufacturer's recommendations (Applied Biosystems). $\beta 2$ microglobulin RNA was used as an internal control. Fold-changes in gene expression were calculated using the standard $\Delta\Delta C_t$ method.

Results

GADF supplementation inhibits spontaneous intestinal polyposis without affecting body weight in APC^{Min/+} mice

During the experiment, the body weight and food and water consumption of all mice were monitored. Food consumption and body weight gain did not differ between the control and GADF groups throughout the study and no mortality was observed in any group (data not shown). GADF treatment did not result in macroscopic changes indicative of toxicity in any organs including the liver, lung and kidney.

GADF supplementation significantly decreased the total number of small intestine tumors by 76%. Control mice developed an average of 16 polyps per animal and GADF treatment decreased this number to 3.9 (Figure 1A). Moreover, as shown in Figure 1B, GADF treatment induced a decrease in the number of small intestine polyps in the proximal, medial and distal sections of 76% (4.6±0.9 versus

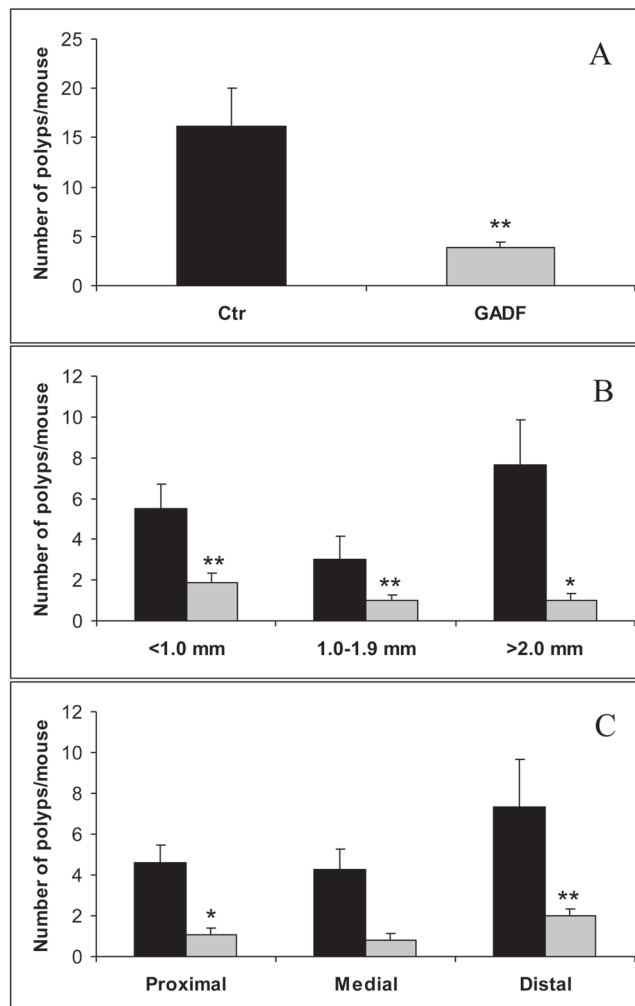


Fig. 1. (A) Total number of polyps/mouse in the small intestine of *Apc*^{Min/+} mice. (B) Number of polyps/mouse in proximal, medial and distal sections. (C) Number of polyps/mouse shown by polyp size distribution (<1 mm diameter polyps, 1–2 mm and >2 mm). Data represented as mean ± SEM (**P* > 0.05) (***P* > 0.01).

1.1±0.3), 81% (4.3±1.0 versus 0.8±0.3) and 73% (7.3±2.4 versus 2.0±0.4), respectively. Analysis of the size distribution of polyps revealed that GADF reduced the occurrence or growth of <1 mm diameter polyps by 65% (5.5±1.2 versus 1.9±0.4), of 1–2 mm by 67% (3.0±1.1 versus 1.0±0.3) and of >2 mm by 87% (7.7±2.2 versus 1.0±0.3) (Figure 1C).

Gene expression profile induced by GADF

To elucidate the underlying mechanisms by which GADF prevents carcinogenesis, we determined the transcriptional profile of the *Apc*^{Min/+} mouse colonic mucosa following GADF treatment using complementary DNA microarrays.

Of the 39000 genes represented on the whole mouse genome complementary DNA microarray, 183 genes were differentially expressed between the control and GADF groups with a 1.5-fold change (FC) or more in expression. Of these 183 differentially expressed genes, 40 genes were upregulated and 143 genes were downregulated. A complete list of these differentially expressed genes is shown in Supplementary Table 1, available at *Carcinogenesis* Online.

This list of differentially expressed genes associated with GADF consumption was subjected to a KEGG molecular pathway analysis using KEGG Mapper to identify any enrichment of genes with specific biological themes. Figure 2 presents the differentially expressed genes detected in the KEGG cell cycle pathway analysis using KEGG Mapper. GADF treatment led to a reduction in the expression of the *Ccnd1* gene, which codes for cyclin D, which in turn is involved in regulating cell cycle progression and drives the G₁/S phase transition.

Moreover, an increase in the expression of a regulator of this protein called *Gadd45a* was detected. The GADD45 protein interacts with many effectors, such as Cdk1/cyclin B, proliferating cell nuclear antigen (which regulates cyclin D/Cdk4,6) and p21, thus mediating cell cycle arrest, differentiation or apoptosis (12). Additionally, the DNA replication pathway represented in KEGG Mapper (data not shown) was also downregulated in the mucosa of *Apc*^{Min/+} mice treated with GADF due to inhibition of the expression of *Pold2* and *Rfc1*, two members of the DNA polymerase complex.

KEGG Mapper analysis also showed the modulation of other genes related to cancer pathways (Figure 3). GADF supplementation downregulated the expression of *Kitl*, which encodes the ligand of the tyrosine-kinase receptor KIT. The ligand for KIT is known as kit ligand or stem cell factor. Furthermore, study of cancer-related pathways showed that the expression of *Tgfb1* was also downregulated. Transforming growth factor-β is a secreted protein that controls a diverse set of cellular processes, including cell proliferation, recognition, differentiation, apoptosis, hematopoiesis, angiogenesis, immune functions, chemotaxis and specification of developmental fate.

As mentioned previously, *Apc*^{Min/+} mice possess a mutation in the *Apc* gene that results in defective Wnt signaling. The representation of the differentially expressed genes detected in the KEGG Mapper Wnt signaling pathway (Figure 4) showed that GADF downregulates the expression of *Csnkle*, which encodes the CKI protein ε. Wnt signaling has long been regarded as the signaling pathway playing a central role in the intestinal epithelial cell differentiated state; however, recent studies have shown that Notch

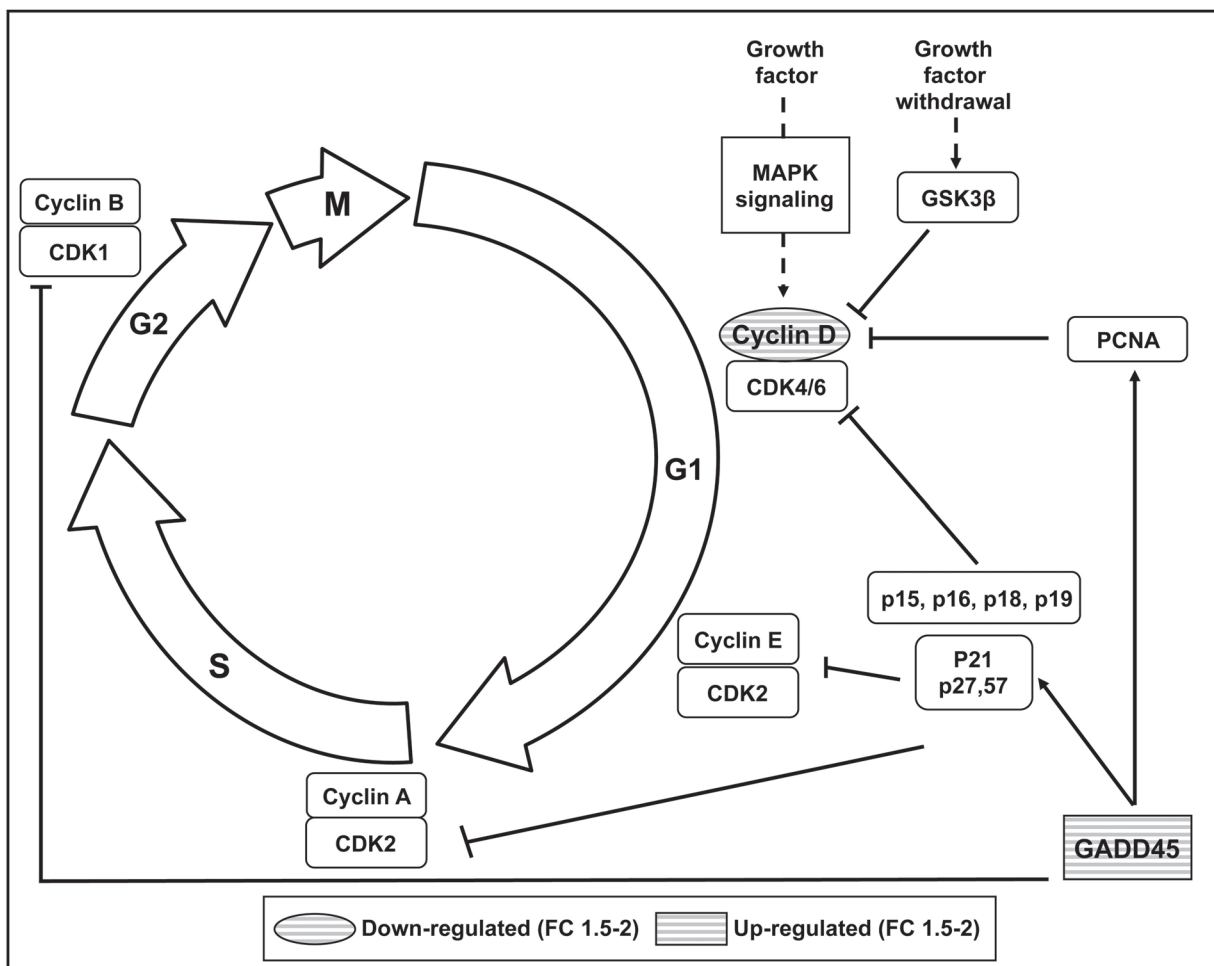


Fig. 2. Adaptation of KEGG cell cycle pathway using KEGG Mapper. Oval pathway members were significantly downregulated and rectangular members were found to be upregulated in the intestinal mucosa of *Apc*^{Min/+} mice treated with GADF. Horizontal lines indicate an FC of between 1.5 and 2.

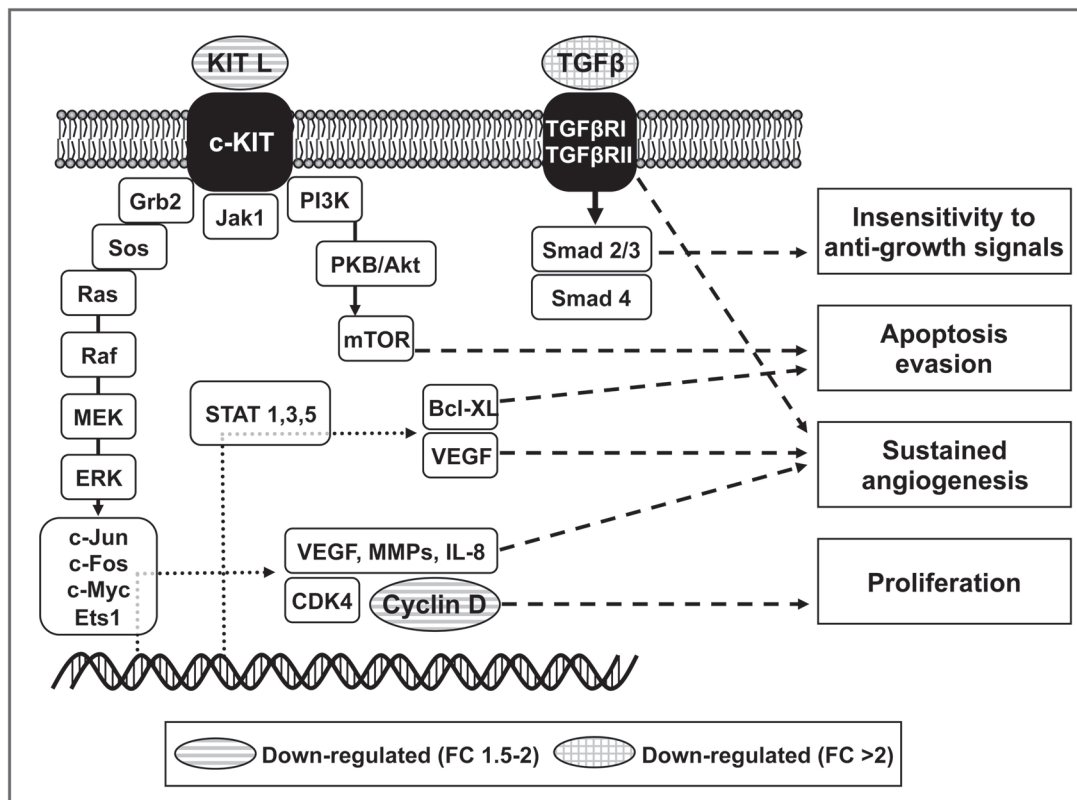


Fig. 3. Adaptation of KEGG pathways in cancer using KEGG Mapper. Ovals represent downregulated genes following GADF supplementation. Horizontal lines indicate an FC of between 1.5 and 2 and vertical lines specify a FC of >2.

signaling is also indispensable for this process (13). In this sense, it is noteworthy that GADF treatment also downregulated *Lfn3* (data not shown), which encodes Fringe, a glycosyltransferase that is involved in the elongation of O-ligands in the Notch pathway (14).

Pathway analysis performed using KEGG Mapper was complemented with an independent analysis by MetaCore to obtain the *P*-value of each pathway. Pathway analysis of significantly modulated genes using MetaCore showed significant changes in maps that contain several canonical pathways. Table I presents the top Maps according to MetaCore, showing the greatest downregulation in the cell cycle, immune system responses and G-protein signaling, whereas cell adhesion was upregulated. In addition to the above-mentioned cell-cycle-associated genes, MetaCore analysis identified upregulation of the PLK3, a protein that has been negatively correlated with the development of certain cancers, including colon cancer (15). MetaCore analysis also revealed the downregulation of immune-system-related genes such as *Lck*, *Nfkbie*, *Cxcr4*, *H2-Ab1*, *Igh*, *Igl-VI* and *Igkv1-117* by GADF. A promoting effect of GADF on enterocytic differentiation was shown by the upregulation of genes related to cell adhesion molecules such as *Ocln*, *Cldn4* and *Epha2* in polarized epithelial cells.

Validation of microarray data by reverse transcriptase–real-time PCR

The changes in messenger RNA expression observed in the microarrays for *Ccnd1*, *Kitl*, *Csnk1e*, *Lfn3* and *Cxcr4* were further validated by reverse transcriptase–real-time PCR (Figure 5). These targets were selected for reverse transcriptase–real-time PCR analysis based on their participation in the pathways that were significantly modulated by GADF supplementation.

Discussion

GADF treatment induced a 76% reduction in intestinal polyposis with respect to the control (Figure 1). Interestingly, GADF exerted a higher antitumoral effect than observed in previous studies in *Apc^{Min/+}* mice

in similar conditions using dietary fiber or other phenolic compounds. For example, administration of 1% dibenzoylmethane reduced the total number of small intestinal tumors by 50% in *Apc^{Min/+}* mice (16) and a reduction in small intestinal tumors of only 25% was observed after intake of a greater content of dietary fiber (17). It is important to note that the decrease in the number of polyps was homogeneous throughout the small intestine. GADF contains a complex mixture of phenolics including monomers of catechins, anthocyanins, flavonols and hydroxycinnamic acids, as well as (epi)catechin oligomers and polymers (PA), all of which are associated with a fiber matrix of both soluble and insoluble polymers such as polysaccharides and lignins that may influence the absorption of the putatively bioactive GADF components. Small phenolics such as phenolic acids and monomeric (epi)catechins that are originally contained within the matrix are absorbed in the small intestine. In previous publications, we described that during transit along the intestinal tract some of the GADF's PA may be partially depolymerized into (epi)catechin monomers and some fermented by the intestinal microbiota and absorbed in the form of smaller phenolic acids (18,19). The fact that GADF exerts its antitumorigenic function homogeneously throughout the intestine could thus be related to the putatively bioactive phenolic compounds embedded in the fiber that are gradually released and absorbed. On the other hand, the size distribution analysis of polyps revealed that GADF inhibits both the appearance and development of intestinal polyps, although the most important inhibitory effect was observed in larger polyps indicating a major inhibition in polyp's progression.

Putative molecular mechanisms underlying the inhibition of intestinal tumorigenesis were investigated by comparison of microarray expression profiles of GADF-treated and non-treated mice. KEGG Mapper analysis mainly showed modifications in cancer-related pathways. Concretely, KEGG cell cycle pathway analysis (Figure 2) suggested that GADF suppresses tumorigenesis in *Apc^{Min/+}* mice by inducing a G₁ cell cycle arrest through cyclin D downregulation and GADD45 upregulation. These results are consistent with previous studies in which dietary supplementation

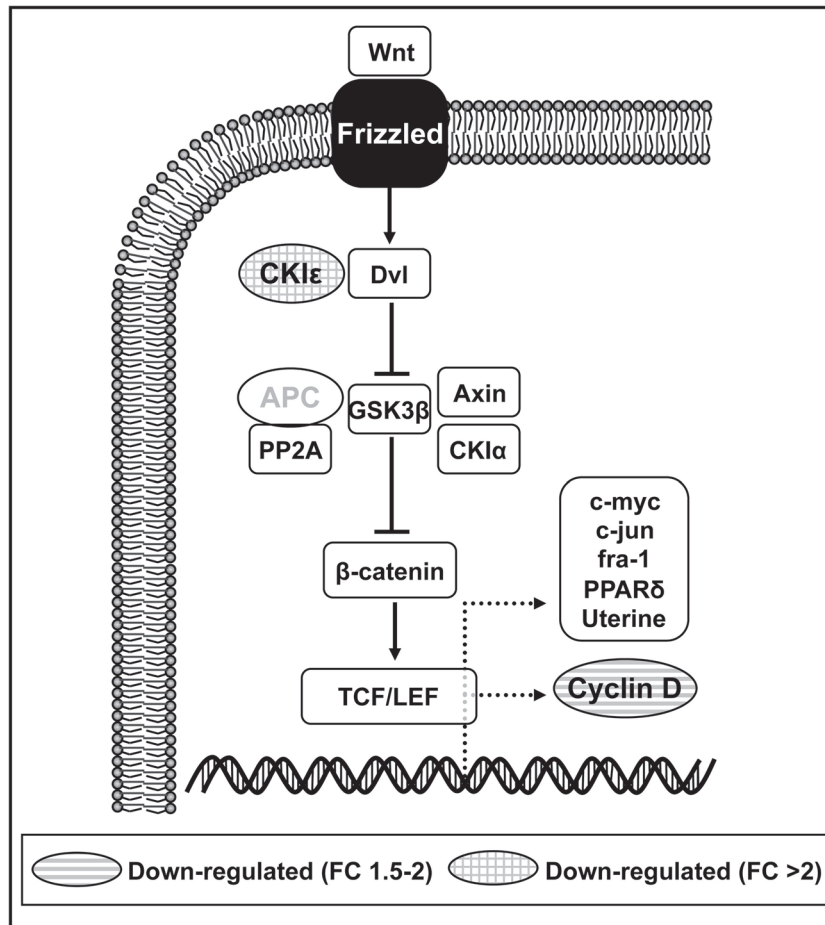


Fig. 4. Adaptation of KEGG Wnt signaling pathway using KEGG Mapper. Ovals represent downregulated genes encoding that protein in $Apc^{Min/+}$ mice following GADF treatment. Horizontal lines indicate an FC in expression of between 1.5 and 2 and vertical lines specify an FC of >2 .

with grape seed extract in $Apc^{Min/+}$ mice was found to downregulate cyclin D1 and upregulate Cip1/p21 in small intestinal tissue samples according to immunohistochemical analysis (20). Likewise, another study reported that grape seed extract upregulates p21, leading to G_1 cell cycle arrest (21). Additionally, inhibition of DNA synthesis, by downregulation of *Pold2* and *Rfc1*, may also be involved in the induction of G_1 arrest in the cell cycle (22). In this regard, it is worth mentioning that GADF produces a change in mucosal epithelial turnover favoring cell differentiation and cell death over proliferation by means of stopping cell cycle progression without affecting cell death processes.

Moreover, downregulation of kit ligand by GADF (Figure 3) may be related to the inhibition of intestinal polyp growth because it has been described that stem cell factor-KIT signaling enhances proliferation and invasion in KIT-positive colorectal cancer cell lines (23). Another downregulated gene, transforming growth factor- β , has been reported to be involved in the progression of colorectal cancer, and therefore a reduction in its expression suggests higher sensitivity to antigrowth signals and a reduction in angiogenesis (24,25).

KEGG Wnt signaling pathway analysis (Figure 4) showed the inhibition of CKI protein ϵ , a positive regulator of β -catenin-driven transcription that is specifically required for the proliferation of breast cancer cells with activated β -catenin (26). Interestingly, an important gene under the transcriptional activation induced by β -catenin/T cell factor/lymphoid enhancer factor, *cyclin D*, was also downregulated by GADF antagonizing the deregulated Wnt signaling pathway in $Apc^{Min/+}$ mice. Recent data indicate that Wnt and Notch signaling might play an equally important role in the maintenance of the undifferentiated state of *Apc*-deficient cells (13). In fact, it

has been reported that Notch signaling occurs downstream of Wnt through β -catenin-mediated transcriptional activation of the Notch ligand Jagged1 (27), suggesting that Notch is an alternative target for the treatment of *Apc*-mutant intestinal polyposis. The inhibition of Fringe, involved in the Notch pathway, suggests that GADF inhibits colon cancer growth through the simultaneous downregulation of Wnt and Notch signaling.

Inflammation and immune system responses have been reported to have dual effects in cancer, either by providing protection from tumor cells or, when inflammation becomes chronic, by promoting tumor growth. Grape phenolic compounds have been implicated in strengthening immune function (28), but their anti-inflammatory and immune-attenuating properties have recently attracted much attention (29,30). These functions may play an important role in $Apc^{Min/+}$ mice because the tumorigenesis initiated by intrinsic defects in pathways regulating cell proliferation, as observed in $Apc^{Min/+}$ mice, is driven by repeated inflammation and excessive immune signaling (31). Accordingly, a study identifying genes involved in tumorigenesis in $Apc^{Min/+}$ mice revealed the upregulation of various immune system and inflammation genes (32). Therefore, in this case, diminished immune signaling by GADF (Table I) may reduce tumor progression. Interestingly, apart from the immuno-attenuating properties of grape phenolics mentioned above, a recent investigation concluded that high fiber intake may be inversely associated with the presence of a cytokine pro-inflammatory profile (33). Therefore, attenuation of the immune response in $Apc^{Min/+}$ mice treated with GADF could be due to the combined effect of soluble phenolics, insoluble PA and other components of the dietary fiber fraction such as polysaccharides and lignins.

Table I. Pathways modified in the colon mucosa of *Apc^{Min/+}* mice by GADF treatment as found in MetaCore

GeneGO maps/modulated pathways	<i>P</i> -value ^a	Significant/total genes ^b
Cell cycle and its regulation (↓)		
Regulation of G ₁ /S transition (part 1) (↓)	0.0010	2 (<i>Ccnd1</i> , <i>Tgfb1</i>)/38
Nucleocytoplasmic transport of cyclin-dependent kinases/cyclins (↓)	0.0023	1 (<i>Ccnd1</i>)/14
ATM/ATR regulation of G ₂ /M checkpoint (↑)	0.0005	2 (<i>Gadd45a</i> , <i>Plk3</i>)/26
Immune response (↓)		
CXCR4 signaling <i>via</i> second messenger (↓)	0.0007	3 (<i>Lck</i> , <i>Nfkbie</i> , <i>Cxcr4</i>)/34
T-cell receptor and CD28 co-stimulation in activation of nuclear factor-κB (↓)	0.0012	3 (<i>H2-Ab1</i> , <i>Nfkbie</i> , <i>Lck</i>)/40
Inducible T-cell co-stimulator pathway in T-helper cell (↓)	0.0017	3 (<i>H2-Ab1</i> , <i>Nfkbie</i> , <i>Lck</i>)/46
Nuclear factor of activated T cells in immune response (↓)	0.0023	4 (<i>H2-Ab1</i> , <i>Nfkbie</i> , <i>Lck</i> , <i>Ig</i>)/51
T-cell receptor signaling pathway (↓)	0.0025	3 (<i>H2-Ab1</i> , <i>Nfkbie</i> , <i>Lck</i>)/52
G-protein signaling (↓)		
G-protein α-q signaling cascades (↓)	0.0007	3 (<i>Rgs2</i> , <i>Plcb4</i> , <i>Nfkbie</i>)/34
Proinsulin C-peptide signaling (↓)	0.0025	3 (<i>Ccnd1</i> , <i>Plcb4</i> , <i>Nfkbie</i>)/52
Cell adhesion (↑)		
Tight junctions (↑)	0.0010	2 (<i>Ocln</i> , <i>Cldn4</i>)/36
Ephrin signaling (↑)	0.0016	1 (<i>Epha2</i>)/45

More significantly modulated pathways in MetaCore using genes with FC > 1.5 and adjusted *P*-value < 0.01.

↑/↓, activation/inhibition of the biological process by GADF.

^a*P*-value that corresponds to the GeneGO map/pathway.

^bRatio between the number of significantly modulated genes by GADF (indicated within parentheses) and the total number of genes per GeneGO map/pathway in MetaCore.

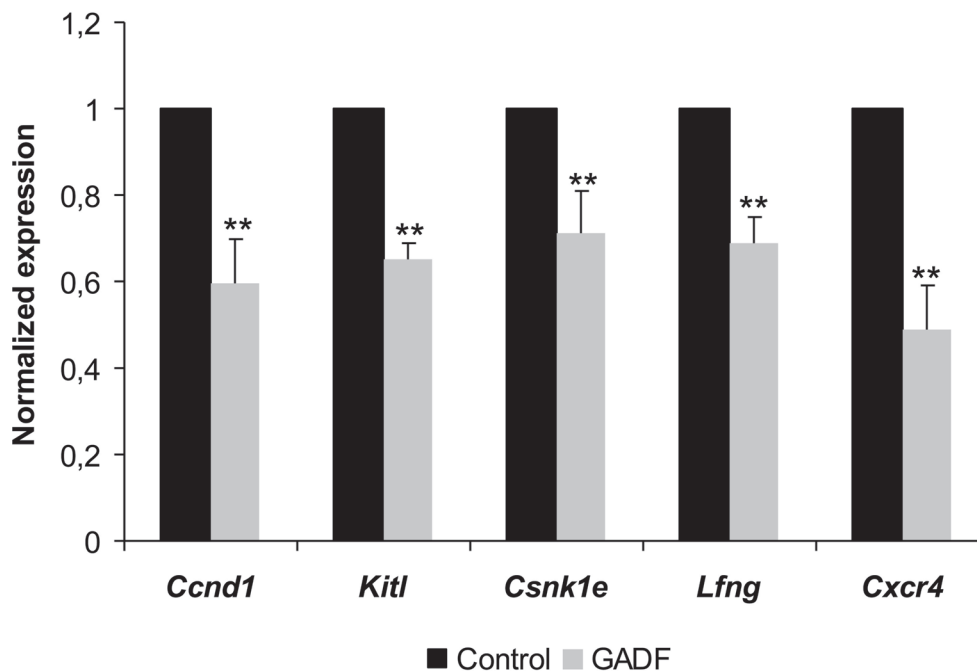


Fig. 5. Validation of genes that were differentially expressed in the colon mucosa of *Apc^{Min/+}* mice after GADF treatment by *reverse transcriptase–real-time PCR*. Data represented as mean ± SEM (***P* > 0.01).

In addition to modulation of the immune response, some of the downregulated immune system/inflammation genes identified in the MetaCore analysis have been associated with tumoral progression. For example, *Cxcr4*, a chemokine receptor specific for stromal cell-derived factor-1, has been reported to be involved in tumorigenicity in breast, pancreatic and colorectal cancer (34–36). Regarding colorectal cancer, the expression of both stromal cell-derived factor-1 and its receptor CXCR4 has been reported to predict lymph node metastasis. Therefore, lower expression of this protein in GADF-fed mice may be related to the inhibition of tumor growth. GADF supplementation also modulated the expression of *Nfkbie*, which has been reported to regulate cell viability and proliferation during transformation (37).

Additionally, *Lck*, a Src-related tyrosine kinase that is expressed in certain tumors such as human colon carcinoma (38) was downregulated in the mucosa of *Apc^{Min/+}* mice treated with GADF.

Interestingly, studies evaluating the consumption of GADF by normal C57BL/6J mice showed many changes in the expression of genes involved in antioxidant activity and xenobiotic metabolism. GADF upregulated genes encoding enzymes implicated in phase I (biotransformation) of the xenobiotic metabolism that convert hydrophobic compounds to more water-soluble moieties, genes from phase II (detoxifying metabolism) that catalyze several conjugation reactions and genes encoding for peroxiredoxins, members of the family of mammalian proteins that neutralize reactive oxygen species (4).

Surprisingly, in *Apc*^{Min/+} mice, GADF had no significant effect on the antioxidant and detoxifying machinery, apart from upregulation of the *Cyp2c54* gene (Supplementary Table 1, available at *Carcinogenesis* Online), which encodes a cytochrome P450, demonstrating the importance of the regulation of cell growth and maintenance functions to the detriment of antioxidant and xenobiotic systems in tumor progression.

We hypothesize that the changes in the gene expression profile induced in the intestinal mucosa of *Apc*^{Min/+} mice treated with GADF and the associated inhibition of spontaneous intestinal polyposis may be a result of the action of phenolic compounds (both soluble and insoluble fiber-like PA) and other components of the dietary fiber fraction. It is likely that the phenolics contained in GADF act through molecular mechanisms such as the modulation of gene expression, as previously reported (39). On the other hand, although the amount of fiber is little (0.75%), it may perhaps act *via* the short chain fatty acids released from their fermentation by the gut microbiota. Short chain fatty acids are mainly used as an energy source by the intestinal epithelium, but they also have been reported to modulate gene expression in several *in vitro* studies (40,41).

In summary, this study shows for the first time that dietary administration of GADF prevents spontaneous intestinal polyposis in the *Apc*^{Min/+} mouse model. The cancer chemopreventive effects of GADF were mainly related to the modulation of cancer progression-related genes, suggesting the induction of G₁ cell cycle arrest and the down-regulation of genes related to the immune response and inflammation, and thus a protective effect against chronic inflammation and excessive immune signaling in *Apc*^{Min/+} mice. The powerful antitumoral effect of GADF may be the result of synergy between the different compounds in the dietary fiber, including soluble and insoluble grape phenolics and insoluble polysaccharides and lignins. The fact that GADF is a by-product of the wine industry makes it of particular economic and health interest. Taken together, our findings show that GADF is a promising nutraceutical for the prevention of colon cancer in high-risk populations.

Supplementary material

Supplementary Table 1 can be found at <http://carcin.oxfordjournals.org/>

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