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RESEARCH PAPER

The fermented non-digestible fraction of common bean (*Phaseolus vulgaris* L.) triggers cell cycle arrest and apoptosis in human colon adenocarcinoma cells

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Abstract Cancer is a leading cause of death worldwide with colorectal cancer (CRC) ranking as the third contributing to overall cancer mortality. Non-digestible compounds such as dietary fiber have been inversely associated with CRC in epidemiological in vivo and in vitro studies. In order to investigate the effect of fermentation products from a whole non-digestible fraction of common bean versus the short-chain fatty acid (SCFAs) on colon cancer cells, we evaluated the human gut microbiota fermented non-digestible fraction (hgm-FNDF) of cooked common bean (*Phaseolus vulgaris* L.) cultivar Negro 8025 and a synthetic mixture SCFAs, mimicking their concentration in the lethal concentration 50 (SCFA-LC_{50}) of FNDF (hgm-FNDF-LC_{50}), on the molecular changes in human colon adenocarcinoma cells (HT-29). Total mRNA from hgm-FNDF-LC_{50} and SCFA-LC_{50} treated HT-29 cells were used to perform qPCR arrays to determine the effect of the treatments on the transcriptional expression of 84 genes related to the p53-pathway. This study showed that both treatments inhibited cell proliferation in accordance with modulating RB1, CDC2, CDC25A, NFKB and E2F genes. Furthermore, we found an association between the induction of apoptosis and the modulation of APAF1, BID, CASP9, FASLG, TNFR10B and BCL2A genes. The results suggest a mechanism of action by which the fermentation of non-digestible compounds of common bean exert a beneficial effect better than the SCFA mixture by modulating the expression of antiproliferative and pro-apoptotic genes in HT-29 cells to a greater extent, supporting previous results on cell behavior, probably due to the participation of other compounds, such as phenolic fatty acids derivatives and biopetides.

Keywords Common bean · SCFA · Non-digestible fraction · Colorectal cancer · PCR-arrays

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Introduction

Colorectal cancer (CRC) is the third leading cause of cancer-related mortality projected to increase in the future (WHO 2011). Cancer was considered a disease of westernized, industrialized countries. However, the situation has changed dramatically, with the majority of the global cancer burden now found in low- and medium-resource countries (Boyle and Levin 2008).

An inverse association between dietary fiber intake and CRC incidence has been shown in epidemiological studies (Dahm et al. 2010). Additionally, research on in vivo and in vitro models has shown the protective role of pulses, primarily due to the presence of indigestible compounds, such as phenolics (condensed tannins, flavonoids and anthocyanins), total dietary fiber (soluble and insoluble), and other secondary metabolites related to the prevention and/or reduction in chronic degenerative diseases (Bazzano

et al. 2001; Beninger and Hosfield 2003; Waldecker et al. 2007). The resulting non-digestible fraction (NDF) (compounds that escaped degradation by intestinal enzymes) may reach the colon and can be fermented by the microbiota, producing short-chain fatty acids (SCFAs) such as acetic, propionic, butyric acids (Delzenne et al. 2003), as well as some phenolic SCFA derivatives formed during the intestinal degradation of phenolic constituents of vegetables and fruits (Waldecker et al. 2007). An optimal antitumor drug would be one which selectively induces apoptosis in tumoral but not in normal cells. There is enough evidence that butyric acid is an agent with such properties, which exerts a chemopreventive effect by inhibiting tumor cell proliferation, as well as inducing apoptosis resulting in a more differentiated phenotype (Sengupta et al. 2006), thus reducing the risk of developing CRC. The protection by SCFA has been studied using in vitro cell cultures, widely used to assess the effect of substances that may induce differentiation, cell cycle arrest, apoptosis and DNA repair in transformed cells (Fu et al. 2004; Li and Li 2006).

It is well known that butyric acid is an important energy source for normal colonocytes ($p53^{+}$) and maintains their growth and proliferation. However, this SCFA prevents the progression of cancer by inhibiting the growth, proliferation and survival of colorectal cells ($p53^{-}$) (Ruemmele et al. 2003). The HT-29 cell line, derived from human colon adenocarcinoma, was established in 1975 (Fogh and Trempe 1975). Genetically, HT-29 cells present typical changes in colorectal tumors, such as APC, K-ras and p53 mutations and the consequent loss of their function, as well as amplification of c-myc (Rodrigues et al. 1990; Choi et al. 2008; Zhang et al. 2009; Wu et al. 2010) and a phenotype of chromosomal instability and aneuploidy. This cell line has been used as a model to investigate the mechanism by which some protective compounds sensitize HT-29 cells to control of proliferation and apoptosis (Beyer-Sehlmeyer et al. 2003; Daly et al. 2005; Campos-Vega et al. 2010).

We previously demonstrated that compounds of common bean can inhibit proliferation and induce apoptosis in both in vivo and in vitro models by modulating the expression of genes involved in cell cycle arrest, cell proliferation and apoptosis (Feregrino-Pérez et al. 2008; Campos-Vega et al. 2010). Our previous study showed that common bean (*Phaseolus vulgaris* L.) Negro 8025 has fermentable substrates, such as the NDF (total dietary fiber, oligosaccharides, resistant starch and phenolic compounds), and the NDF was submitted to gut in vitro fermentation producing compounds that showed chemoprotective effect by inducing DNA fragmentation (apoptosis) and inhibiting the HT-29 cell survival (Cruz-Bravo et al. 2011). This investigation extends our previous study in evaluating the effect of the fermented non-digestible fraction (FNDF) of common bean (*P. vulgaris* L.) cultivar Negro 8025 on the modulation of p53-pathway-related gene expression in HT-29 cells, as well as showing that a complex of food compounds may exert a major effect in mutated p53 cells compared to chemoprotective agents separately. Also, we pretend to extend the knowledge on the mechanism of action of SCFA (mimicking their physiological produced concentrations during human gut microbiota fermentation) on colon cancer cells.

Materials and methods

Dry bean seeds

Beans of cultivar Negro 8025 were harvested in 2007 at the Bajio Experimental Station of the National Research Institute for Forestry, Agriculture and Livestock (INIFAP), located in Celaya, Guanajuato, Mexico. The seeds, cooked bean and the NDF were stored at 4 °C and protected from light.

Chemicals

Butyrate was purchased from Fluka™ (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada; ≥99 %). Acetate, propionate (≥99.6 %) and other chemicals were purchased from J. T. Baker™ (Mexico City, Mexico). Protease, α-amylase and amyloglucosidase were obtained from Sigma (Sigma-Aldrich, Canada Ltd.). Other chemicals were purchased from J. T. Baker™ (Mexico).

Non-digestible fraction extraction

Beans were cooked using a "traditional" cooking process according to the method of Aparicio-Fernandez et al. (2005). The AOAC method 991.43 (AOAC 2002) was used to obtain total dietary fiber (TDF) considered here as the NDF. Briefly, 1 g of each sample (uncooked and cooked) was placed in different Erlenmeyer flasks with 50 mL phosphate buffer (0.08 mol/L, pH 6) added and adjusted to pH 6 with 0.375 M HCl or 0.275 M-NaOH. Samples were placed in a water bath at 100 °C, and 0.1 mL a-amylase added to each and incubated for 30 min with manual stirring every 5 min. The flasks were cooled rapidly and the samples adjusted to pH 7.5. After the addition of 0.1 mL of protease (5 mg/mL phosphate buffer), samples were placed in a water bath at 60 °C for 30 min. Samples were cooled, and the samples' pH was adjusted to pH 4. After pH adjustment, samples were placed in the water bath at 60 °C for 30 min, and 0.3 mL of amyloglucosidase was added. Samples were incubated for 30 min under constant

agitation. Then, 95/100 mL ethanol at room temperature was added at a 1:4 sample/ethanol ratio, and the mixture left at room temperature for 24 h. Samples were filtered at a constant weight, and residues were washed tree times with 10 mL of distilled water. The residues were then placed in an oven at 90 °C for 2 h and weighed. The TDF was determined gravimetrically. After the results were corrected for protein and ash, it was considered as NDF. At least, three determinations of each treatment were conducted. To quantify insoluble dietary fiber (IDF), the ethanol was not added. The soluble dietary fiber (SDF) was calculated by subtracting the IDF proportion from TDF.

In vitro lower gastrointestinal fermentation

This study utilized a human gut flora fermentation method to estimate the effects of NDF digestion in the colon representing simulated large bowel model. It provides useful data to form hypotheses for in vitro studies. In vitro fermentation was done following by Campos-Vega et al. (2009) method. Fermentations were performed in duplicate for NDF extract from cooked bean in a water bath at 37 °C. Raffinose was used as a positive control for fermentable sugar under the same conditions. Human gut flora was collected from fecal sample supplied by one healthy volunteer who had no previous history of gastrointestinal disorders and had not undergone antibiotics therapy within 3 months prior to the study. Sterile tubes (15 mL) were filled with 9 mL of sterile basal culture medium containing (g/L): peptone water, 2; yeast extract, 2.0; NaCl, 0.1; K_2HPO_4, 0.04; KH_2PO_4, 0.04; $MgSO_4{\cdot}7H_2O$, 0.01; $CaCl_2{\cdot}2H_2O$, 0.01; $NaHCO_3$, 2; cysteine HCl, 0.5; bile salts, 0.5; Tween-80, 2 mL; and hematin, 0.2 g (diluted in 5 mL of NaOH). Sealed tubes were maintained under a headspace containing H_2–CO_2–N_2 (10:10:80, v/v/v); O_2-free for 24 h. Human gut flora slurries were prepared by homogenizing 2 g of fresh stools with 18 mL of 0.1 M-sodium phosphate buffer, pH 7. The tubes containing basal culture medium were inoculated with 1 mL of fecal slurries, and the polysaccharides (100 mg) were added after inoculation, except for blanks. The samples were vortexed for 30 s and placed in a water bath at 37 °C. During fermentation, the pH of the sample and SCFAs production was assessed at 6, 12 and 24 h. Fermented extract was centrifuged (Hermle Z 323 K, Germany) at 4,500 rpm for 15 min at 4 °C. The supernatant (FE-hgf) was transferred to 1.5 mL tubes and placed in a freezer at −70 °C until bioassay.

Cell culture

Beans were cooked, and the NDF was extracted in order to perform the in vitro fermentation as described previously (Cruz-Bravo et al. 2011). HT-29 cells from a human colon adenocarcinoma of a Caucasian female containing mutated p53 purchased from the American Type Culture Collection (ATCC) were grown and maintained in McCoy's 5a medium (ATCC) supplemented with 10 % fetal bovine serum (FBS, Gibco^{TM}, Grand Island, NY, USA) and 1 % antibiotic-antimycotic (Gibco^{TM}, Grand Island, NY, USA) at 37 °C under 5 % CO_2 air atmosphere. Subculture of HT-29 cell line was performed by enzymatic digestion (trypsin/EDTA solution: 0.05/0.02 %) (Sigma-Aldrich, Canada Ltd.). HT-29 cells were cultured in 24-well plates at a density of 5×10^4 cells/well under the growth conditions indicated above for 24 h. The medium was changed by adding McCoy's 5a medium supplemented with bovine serum albumin (BSA Sigma–Aldrich(tm), Canada Ltd.) plus different concentrations (5, 10, 15, 20, 25, 30, 35 %) of 100 % fermented NDF (previously sterilized by filtration). After incubation, cells were harvested. Hemocytometer counts were performed, and growth inhibition rate was plotted to determine lethal concentration 50 (LC_{50}) value. Mc-Coy's 5a medium containing 0.5 % BSA was also added to control cell culture. All data points were performed in duplicate, and each experiment was repeated independently at least twice for statistical analysis.

mRNA extraction and cDNA synthesis

According to our previous results (Cruz-Bravo et al. 2011), we considered the LC_{50} of hgf-FNDF (hgm-FNDF-LC_{50}) and a mixture of synthetic SCFAs mimicking their concentration in the hgm-FNDF-LC_{50} (equivalent to 7.36, 0.33, and 3.31 mmol of acetic, propionic and butyric acids, respectively) to treat HT-29 cells in order to observe the effect of the fermented NDF extract from bean versus a mixture of SCFA on a colorectal cell line. The extraction of total mRNA from 2×10^6 treated and untreated cells was carried out using the RNeasy Mini Kit according to the Qiagen's protocol. All mRNA samples were examined for the quality of the nucleic acid, that is, absence of DNA and mRNA degradation by denaturing agarose gel electrophoresis. For complementary DNA (cDNA) synthesis, 230 ng mRNA was reverse transcribed and amplified with the SMART PCR cDNA synthesis kit and the Advantage cDNA PCR kit (CLONTECH Laboratories). The first-strand cDNA synthesis was done as specified in the manufacturers' user manual and included total mRNA, the CDS synthesis primer, the SMART II oligonucleotide and Superscript II. The double-stranded cDNA was amplified with Platinum Taq DNA polymerase (Invitrogen), PCR primer (from the SMART PCR cDNA synthesis kit) using the following conditions: 95 °C 2 min and then 15 cycles of 94 °C-30 s/55 °C-30 s/72 °C-6 min.

Table 1 Up-regulated genes by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments compared with untreated HT-29 cells

Symbol	GenBank	Biological function	Fold regulation	
			hgm-FNDF-LC_{50}	SCFA-LC_{50}
Apoptosis				
APAF1	NM_001160	Induction of apoptosis	108.38	3.24
BID	NM_001196	Induction of apoptosis	32.00	19.12
CASP2	NM_032982	Induction of apoptosis	1.07	3.47
CASP9	NM_001229	Induction of apoptosis	12.73	3.88
EI24	NM_004879	Induction of apoptosis	8.57	–
FASLG	NM_000639	Induction of apoptosis	2.13	4.55
TNF	NM_000594	Induction of apoptosis, cell proliferation, inflammatory response	3.63	–
TNFRSF10B	NM_003842	Induction of apoptosis	8.46	3.11
GML	NM_002066	Induction of apoptosis	2.27	1.64
Regulation of cell cycle				
ATR	NM_001184	Negative regulation of cell cycle, DNA repair	4.63	3.15
BAI1	NM_001702	Inhibition of angiogenesis and growth	–	6.90
CCNE2	NM_057749	Cell cycle checkpoint	7.94	–
CCNG2	NM_004354	Cell cycle checkpoint	3.86	5.30
CHEK2	NM_007194	Cell cycle checkpoint	1.64	3.52
E2F3	NM_001949	Regulation of cell cycle	–	3.20
GADD45A	NM_001924	Cell cycle arrest, DNA repair	1.18	1.42
NF1	NM_000267	Negative regulation of cell cycle	4.66	–
PCBP4	NM_020418	Negative regulation of cell cycle, induction of apoptosis	–	2.23
RB1	NM_000321	Cell cycle checkpoint	3.36	2.26
RPRM	NM_019845	Negative regulation of cell cycle	3.58	7.00
SESN1	NM_014454	Negative regulation of cell cycle	12.38	28.18
SIRT1	NM_012238	Induction of apoptosis, negative regulation of cell cycle	29.86	14.19
TSC1	NM_000368	Negative regulation of the cell cycle	1.78	10.32
Cell proliferation				
IL6	NM_000600	Induction of inflammatory response and cell proliferation	2.14	2.69
KRAS	NM_004985	Cell proliferation	2.81	3.96
MDM4	NM_002393	Negative regulation of cell proliferation	6.06	3.55
KAT2B	NM_003884	Inhibition of cell growth	3.12	12.79
PPM1D	NM_003620	Cell growth and anti-apoptosis	4.44	–
PRKCA	NM_002737	Cell proliferation	6.36	4.65
TNFRSF10D	NM_003840	Anti-apoptosis, cell survival	1.82	4.49
DNA repair				
MLH1	NM_000249	DNA repair genes	2.06	3.31
MSH2	NM_000251	DNA repair genes	2.57	2.31

Results were normalized to housekeeping genes, and values represent the degree of changes in mRNA for treated HT-29 cells relative to untreated HT-29 cells. $P = 0.05$ compared with the control

– Not applicable

Quantitative real-time PCR (qPCR)

For quantitative determination of transcripts, 102 μL of diluted cDNA (100 ng/μL) was mixed with RT^2 real-time™ SYBR Green/ROX PCR mastermix (PA-012, SA-Biosciences, Frederick, MD, USA), and the RT^2 Profiler™ PCR array human p53 signaling pathway (96 well) (PAHS-027A, SABiosciences, USA) was used as specified in the manufacturers' user manual. The human p53 signaling pathway RT^2 Profiler™ PCR array shows the expression of 84 genes related to p53-mediated signal transduction involved in the processes of apoptosis, cell cycle, cell growth, proliferation, differentiation and DNA repair plus three housekeeping genes (B2M, GAPDH and ACTB).

Real-time PCR was done using the Mx3005P QPCR system (Stratagene, La Jolla, CA, USA) with the following protocol: 95 °C 10 min and then 40 cycles of 95 °C 15 s/60 °C 1 min. Data were evaluated with the MxProsoftware (Stratagene). To correct for well-to-well fluorescent fluctuations, normalization of the SYBR Green-dsDNA complex signal to the passive reference dye ROX (included in the SYBR Green PCR mastermix) was performed. Relative gene expression levels were calculated by the comparative Ct method including normalization to the constitutively expressed gene and to a control sample. Data were analyzed by PCR array data analysis web portal (http://www.sabioscience.com/pcr/arrayanalysis.php) based on the $\Delta\Delta C_t$ method with normalization of the raw data to housekeeping genes. Excel-based data analysis template was used. Sequences as potential target genes were considered if the fold regulation between FNDF-LC_{50}- or SCFA-LC_{50}-treated and untreated HT-29 cells was greater than 2, following the instruction from data analysis web portal. Additionally, testing for overrepresentation of functional categories was carried out using database for annotation, visualization and integrated discovery (DAVID) tools (da Huang et al. 2009). Categories were analyzed including gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) database.

Results and discussion

qPCR arrays were performed to profile the modulation of gene expression of 84 genes related to p53 pathway in treated and untreated HT-29 cells. Our results are presented as >twofold up- or down-regulated genes between hgm-FNDF-LC_{50}- or SCFA-LC_{50}-treated and untreated cells (Tables 1, 2) according to the data analysis obtained from the manufacturer's software. However, we also considered some genes that were <twofold up- or down-regulated since their mRNA levels could be functionally relevant (Iacomino et al. 2001).

Transcriptional regulation of cell cycle-related genes

Tumor can progress by defects in many molecules regulating cell cycle, such as p53 and other molecules controlling cell cycle progression. Rodrigues et al. (1990) showed that HT-29 cells have mutations that overproduce mutant p53, function associated with immortalization in vitro and in vivo development of cancer (Peralta-Zaragoza et al. 1997; Iacomino et al. 2001). Interestingly, this gene was the most down-regulated by both hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments in our study (−84.45- and −42.62-fold, respectively; Table 2). A similar effect was observed by Campos-Vega et al. (2010) after treatment by the fermented polysaccharide extract from common bean cultivar Bayo Madero in HT-29 cells, suggesting the modulation of mutated p53 as an inhibiting mechanism for non-digestible compounds of common bean in colon adenocarcinoma cells, and probably dependent of the cultivar since the effect of the hgm-FNDF-LC_{50} on this gene expression was in a lesser extent (−4.8).

On the other hand, cell survival in cancer progression is generally supported by several mechanisms, such as proliferation, lack of apoptosis, differentiation and DNA repair. During the transition from G1 to S phase, different substrates can be targeted by the cyclin-CDK complexes, such as the tumor suppressor Rb, which is associated with the transcription factor E2F. When the protein coded by its gene (pRb) is phosphorylated, E2F is released during the G1 phase and then participates in the transcription of several genes involved in cell cycle progress (Iacomino et al. 2001; Bai and Merchant 2001). In this study, the RB gene was induced (3.36- and 2.26-fold by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments, respectively; Table 1), while E2F was inhibited by both treatments (−5.54- and −5.40-fold by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments, respectively; Table 2) suggesting their participation in cell cycle control.

Furthermore, CDK4 that forms an active complex with Cyclin-D responsible for the first phosphorylation of tumor suppressor Rb in G1 (Zhang and Dean 2001) was down-regulated by both hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments (−1.19- and −1.07-fold, respectively; Table 2), suggesting a possible cell cycle arrest in G1–S phase. Phosphorylation and dephosphorylation of CDK proteins are other regulatory mechanisms for cell cycle progression. CDC25 removes inhibitory phosphates from specific tyrosine and threonine residues within the ATP-binding domain of the CDK proteins, thus activating these kinases. Overexpression of CDC25A plays a critical role in establishing transformed phenotypes, generally characterized by unrestricted cell cycle progression and/or suppressed cell death. The overexpression of CDC25A could also affect the responsiveness of cancer cells to oxidative and/or genotoxic stresses caused by cancer therapies (Iavarone and Massagué 1997). As cells approach M-phase (Fig. 1), the phosphatase CDC25A is activated and thus activates CDC2 (CDK1). In animals, CDC2 associates with an A- or B-type cyclin. The CDC2-cyclin B complex is able to establish a feedback amplification loop that efficiently drives the cell into mitosis (Zou et al. 2001). Its formation depends on the synthesis and proteolysis of the cyclin subunit and must enter the nucleus so that CDC2 is phosphorylated at threonine 161 by a kinase to be active (Mailand et al. 2002). On the other hand, CDC25 antagonizes the inhibitory phosphorylation of tyrosine 15 and

Table 2 Down-regulated genes by hgm-NDF-LC_{50} and SCFA-LC_{50} treatments compared with untreated HT-29 cells

Symbol	GenBank	Biological function	Fold regulation	
			hgm-FNDF-LC_{50}	SCFA-LC_{50}
Regulation of apoptosis				
BAX	NM_004324	Induction of apoptosis	−2.36	−1.57
BCL2	NM_000633	Anti-apoptosis	−1.25	−1.37
BCL2A1	NM_004049	Anti-apoptosis	−5.54	−1.44
BIRC5	NM_001168	Anti-apoptosis	−24.42	–
CRADD	NM_003805	Induction of apoptosis	−4.26	−2.12
ESR1	NM_000125	Induction of apoptosis, Inhibition of cell proliferation	−12.04	−11.82
FADD	NM_003824	Induction of apoptosis	−2.11	−1.54
LRDD	NM_018494	Induction of apoptosis	−2.23	−1.56
TP53AIP1	NM_022112	Induction of apoptosis	−4.82	−1.88
TP63	NM_003722	Induction of apoptosis	−1.02	−3.93
TRAF2	NM_021138	Anti-apoptosis	−2.85	−1.63
Regulation of cell cycle				
BTG2	NM_006763	Negative regulation of cell cycle and proliferation	−2.14	−1.39
CCNB2	NM_004701	Cell cycle checkpoint	−1.49	−1.07
CCNH	NM_001239	Control of transcription and cell cycle	−1.69	−4.45
CDC2	NM_001786	Regulation of cell cycle	−3.16	−9.15
CDK4	NM_000075	Cell proliferation	−1.19	−1.07
CDKN2A	NM_000077	Cell cycle arrest	−3.81	–
CHEK1	NM_001274	Cell cycle checkpoint	−10.48	−1.04
E2F1	NM_005225	Regulation of cell cycle, apoptosis	−5.54	−5.40
E2F3	NM_001949	Regulation of cell cycle	−3.25	–
IFNB1	NM_002176	Negative regulation of cell proliferation	−3.18	−2.35
IGF1R	NM_000875	Regulation of cell cycle	−2.16	–
MYOD1	NM_002478	Negative regulation of cell cycle, differentiation	−2.50	−1.16
NF1	NM_000267	Negative regulation of cell cycle	–	−3.69
SESN2	NM_031459	Negative regulation of cell cycle	−3.23	−2.13
TADA3L	NM_006354	Induction of apoptosis, negative regulation of cell cycle	−2.68	−1.54
TP53	NM_000546	Induction of apoptosis, negative regulation of cell cycle	−84.45	−42.62
TP73	NM_005427	Induction of apoptosis, negative regulation of cell cycle	−10.27	−2.63
WT1	NM_000378	Negative regulation of the cell cycle	−6.63	−1.79
Cell proliferation				
CDC25A	NM_001789	Control of cell proliferation	−14.12	−5.25
CDC25C	NM_001790	Control of cell proliferation	−6.32	–
EGR1	NM_001964	Other genes related to cell growth, proliferation and differentiation	–	−2.30
HK2	NM_000189	Cell proliferation	−2.79	−3.17
MDM2	NM_002392	Negative regulation of cell proliferation, induction of apoptosis	−24.42	−30.55
MYC	NM_002467	Cell proliferation	−5.54	−2.65
NFKB1	NM_003998	Cell proliferation, anti-apoptosis	−25.11	−12.41
PRC1	NM_003981	Cell proliferation	−4.63	−1.05
STAT1	NM_007315	Regulation of apoptosis and cell proliferation	−3.39	−2.61
Cell growth and DNA repair				
BRCA1	NM_007294	Tumor suppressor gene, DNA repair	−20.11	−8.19
BRCA2	NM_000059	Tumor suppressor gene, DNA repair	−6.41	−5.25
DNMT1	NM_001379	Regulation of cytosine methylation	−17.39	−32.30
PPM1D	NM_003620	Cell growth and anti-apoptosis	–	−2.09

Table 2 continued

Symbol	GenBank	Biological function	Fold regulation	
			hgm-FNDF-LC_{50}	SCFA-LC_{50}
XRCC5	NM_021141	DNA repair genes	−2.23	−4.67

Results were normalized to housekeeping genes, and values represent the degree of changes in mRNA for treated HT-29 cells relative to untreated HT-29 cells. $P = 0.05$ compared with the control

– Not applicable

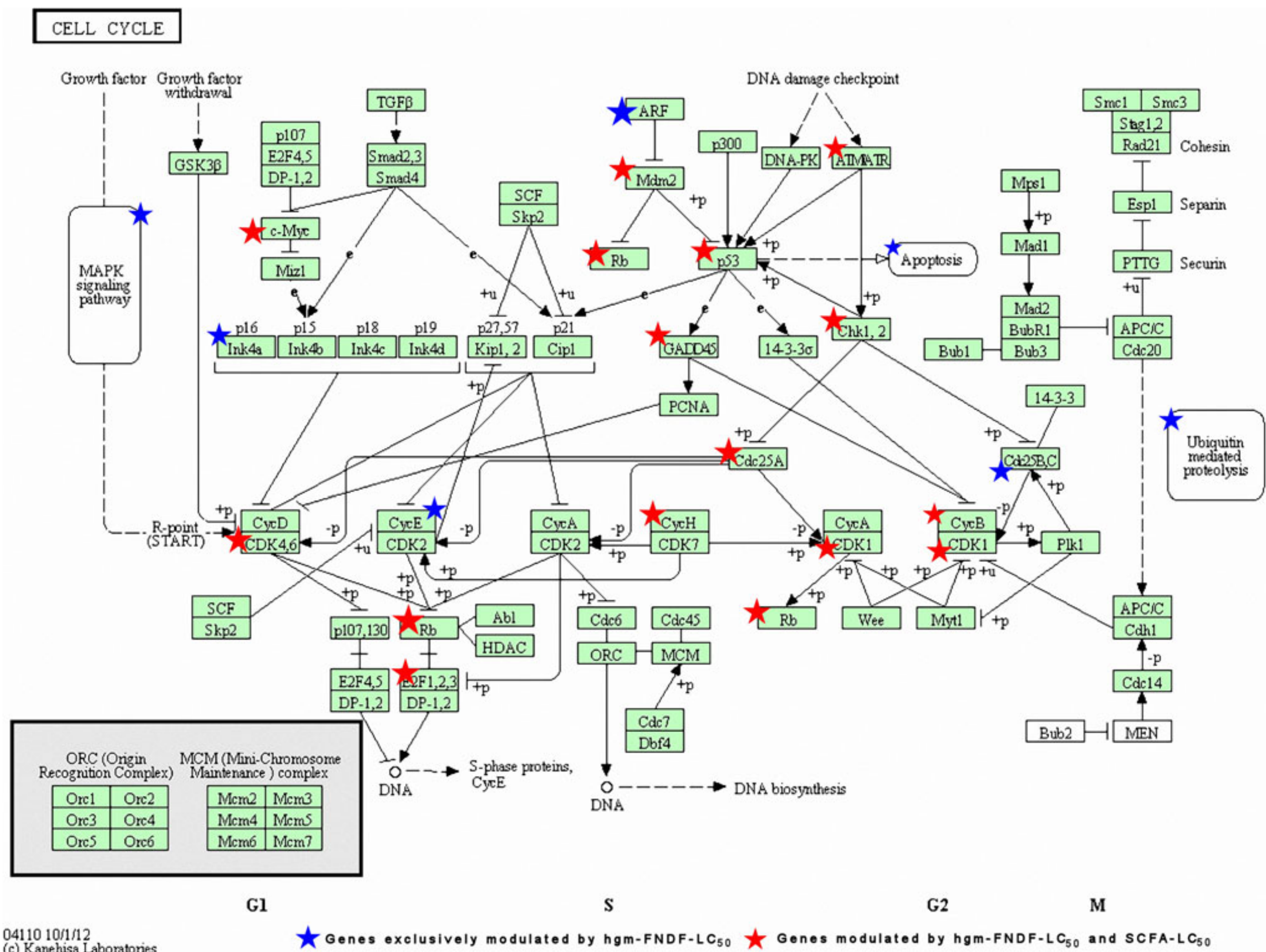

Fig. 1 Interaction among differentially modulated genes in the cell cycle biochemical pathway, using KEGG, after hgf-FNDF-LC_{50} and SCFA-LC_{50} treatments

threonine 14 of CDC2 at the G2/M boundary (Fesquet et al. 1993). Our results showed that hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments down-regulated both CDC25A (−14.12- and −5.25-fold, respectively; Table 2) and CDC2 (−3.16- and −9.15-fold, respectively; Table 2), suggesting that these treatments may have induced the cell cycle arrest in both G1–S and G2–M phases. Both treatments also down-regulated other genes (Table 2) related to proliferation, such as the transcription factor for cytokines STAT1 (−3.39- and −2.61-fold, respectively), PRC1 (−4.63- and −1.05-fold, respectively), which encodes a cell cycle protein that plays important roles during cytokinesis (Draetta and Eckstein 1997), as well as Hexokinase II (HK2) (−2.79- and −3.17-fold, respectively), involved in glycolysis, an essential process to maintain cell proliferation (34). Similar modulation was observed when HT-29

cells were treated with a fermented polysaccharide extract of common bean (*P. vulgaris* L.) cultivar Bayo Madero (Campos-Vega et al. 2010), supporting the suggesting inhibiting effect on cell cycle progression by derivative compounds of common bean fermentation.

SIRT1 belongs to sirtuin proteins with NAD+-dependent deacetylase activity; since this deacetylase can down-regulate the activity of NFκB, it can also be considered as anti-proliferative (Wolf et al. 2011). Furthermore, SIRT1 has been shown to inhibit colon cancer growth in mouse model (Yeung et al. 2004) and may be stimulated by polyphenols in HT-29 cells (Firestein et al. 2008). In our previous report, we showed that the hgm-FNDF has a residual content of phenolics (Cruz-Bravo et al. 2011), which is probably having a role in the effect by hgm-FNDF-LC_{50}. In our study, SIRT1 was up-regulated by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments (29.86- and 14.19-fold, respectively; Table 1), thereby implying that this gene may be a part of the mechanism by which the hgm-FNDF-LC_{50} inhibits HT-29 cells survival.

Transcriptional regulation of apoptosis and DNA repair-related genes

Programmed cell death (apoptosis) can be triggered by several molecules (Fig. 2), such as tumor necrosis factor-alpha (TNF-α), a multifunctional proinflammatory cytokine considered as anti-cancer agent, playing important roles in several physiological and pathological processes by modulating growth arrest, differentiation and apoptosis (de Boer et al. 2006). For instance, TNFR1 (TNF Receptor-1) expressed by all human tissues is the major signaling receptor for TNF-α; when bound by TNF-α, this molecule forms a trimer and recruits an adaptor protein, TRADD, initiating the caspase cascade resulting in apoptosis (Basile et al. 2003). This receptor was up-regulated after both hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments (8.46- and 3.11-fold, respectively; Table 1); thereby representing one of the pathway, the colon adenocarcinoma cells under study can trigger cell death.

FASLG (up-regulated 2.13- and 4.55-fold by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments, respectively; Table 1) is a ligand that can also induce apoptosis by

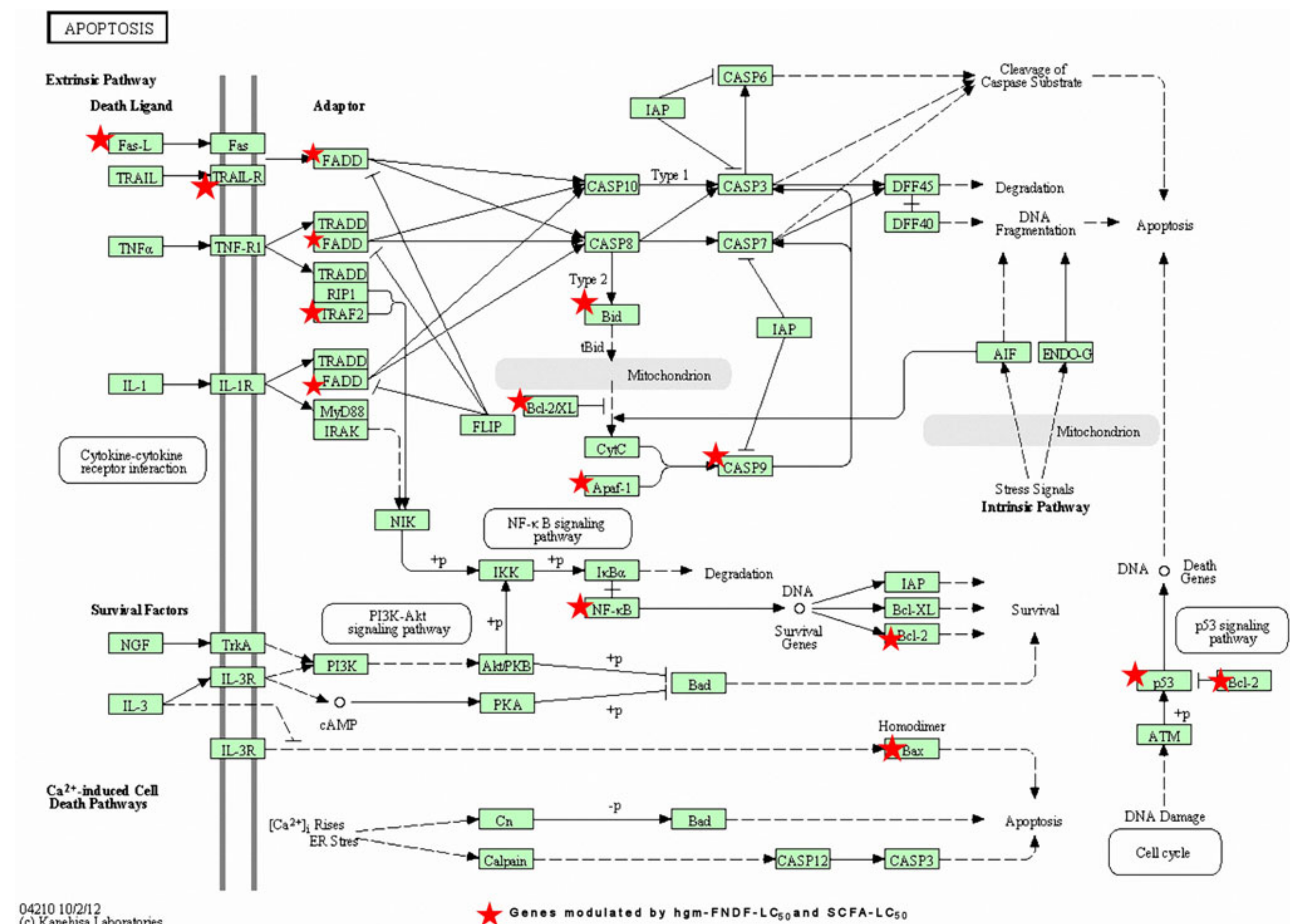

Fig. 2 Interaction among differentially modulated genes in the apoptosis biochemical pathway, using KEGG, after hgf-FNDF-LC_{50} and SCFA-LC_{50} treatments

binding to its receptor Fas on the surface of CRC cells (Yang et al. 2011). Some compounds, such as sodium butyrate, sensitize cancer cells (including HT-29) to p53-independent, Fas-mediated apoptosis (Bonnotte et al. 1998; Yang et al. 2012), suggesting this pathway as a mechanism for the treatments on the induction of extrinsic apoptosis by the treatments evaluated in this study. However, apoptosis may also be carried out through the mitochondrial pathway by several molecules such as the pro-apoptotic BID (up-regulated 32- and 19.12-fold by both treatments, respectively; Table 1). BID modifies the mitochondria by interacting with Bak and stimulates the opening of the mitochondrial membrane releasing Cytochrome-C to form the APAF-1 complex called apoptosome activating caspase 9 (CASP9), which triggers executioner caspases leading to apoptosis (Giardina et al. 1999). APAF-1 (the highest up-regulated gene by hgm-FNDF-LC_{50}) is a key regulator of the mitochondrial apoptosis, and its transcriptional expression is lost or reduced in human colon adenocarcinoma cells, which implicates a poor prognosis, including poor differentiation, tumor invasion and metastasis (Henderson et al. 2003). Hence, therapies stimulating the expression of this gene are highly desirable. In this study, the up-regulation of both APAF-1 and CASP9 (108.38 and 3.24, respectively; 12.73- and 3.38-fold, respectively; Table 1) by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments suggests their role in the induction of intrinsic apoptosis.

Another gene involved in apoptotic induction and cell cycle arrest is EI24 also known as PIG8. It is a DNA damage response gene located on human chromosome 11q23 in a region frequently altered in several human malignancies, and it suppresses cell growth by inducing apoptotic cell death (Paik et al. 2007). Although this gene is generally considered as a target of p53 transcription factor, it was up-regulated after hgm-FNDF-LC_{50} treatment (8.57-fold; Table 1) in the mutated p53-HT29 cells. Burns et al. (2001) found that under an apoptotic stimulation, EI24 mRNA level was not dependent on the presence of p53 in spleen of mice. Therefore, p53 is probably not playing a key role in apoptosis. Hence, we suggest that HT-29 cells death can be induced p53 independent.

In contrast, apoptosis can be inhibited by anti-apoptotic genes, such as BCL2A1 (suppressed 5.54 and 1.44 fold after hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments, respectively. Table 2), which blocks the caspase cascade by interacting with BAX. Furthermore, BCL2A1 expression can be induced by NFκB, and it contains a binding site for NFκB stimulating its activity (Chen et al. 2004). The inhibition of this gene by antitumor compounds in foods including common bean has been previously demonstrated (Feregrino-Pérez et al. 2008; Campos-Vega et al. 2010).

The complex TNF-α and TRADD can also bind to TRAF2 protein, to transmit the TNF-α signal through NFκB (widely known to support cancer progression). The protein encoded by the latter is an important regulator in cell fate decisions by the programmed cell death and proliferation control, and is critical in tumorigenesis (Basile et al. 2003). Furthermore, it can be activated by exposure of cells to inflammatory cytokines, growth factors, oxidant-free radicals and other stimuli. Complete and persistent inhibition of NFκB has been linked directly to apoptosis and delayed cell growth (H-Zadeh et al. 2006; Liu et al. 2010). Down-regulation of both TRAF2 and NFκB by hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments (−2.85- and −1.63-fold, respectively; −25.11- and −12.41-fold, respectively; Table 2) implies that these genes may be involved in the proliferation inhibition of HT-29 cells. The apoptotic process can also be inhibited by other molecules called inhibitor of apoptosis proteins (IAPs), such as BIRC5 (also known as Survivin), (down-regulated 24.42-fold after hgm-FNDF-LC_{50} treatment; Table 2), which may also be under-expressed in HT-29 cells in response to butyrate (Daly et al. 2005).

Nevertheless, we found contradictory results in this study, such as the induction of genes that support cell survival (CCNE2, CCNG2, E2F3, IL6, KRAS; Table 1), and the suppression of BAX and ESR1 (Table 2) and the transcriptional expression of DNA repair genes, such as MSH2 (2.57- and 2.31-fold), MLH1 (2.06- and 3.31-fold), ATR (4.63- and 3.15-fold) and GADD45A (1.18- and 1.42-fold) were also induced by both hgm-FNDF-LC_{50} and SCFA-LC_{50} treatments, respectively. These results are comparable with those reported previously in CRC cell lines treated with butyrate and other chemopreventive compounds (Daly et al. 2005; Campos-Vega et al. 2010). These results correspond to conditions where cell responds to cytotoxic drugs by activating a physiological cell growth inhibition, or trying to recover from it, as suggested between pro- and anti-apoptotic events (Iacomino et al. 2001). Moreover, we observed up-regulation of some genes only after treatment with hgm-FNDF-LC_{50}, not so with the SCFA-LC_{50} (Tables 1, 2). In our previous report (Cruz-Bravo et al. 2011), we showed that the hgm-FNDF-LC_{50} also contains phenolic compounds and probably some biopeptides, suggesting a synergistic effect influenced by the whole hgm-FNDF-LC_{50} on the HT-29 cells. Hence, diet composition may influence the CRC progression through several mechanisms (Johnson 2004).

Biological significance

Gene ontology (GO) term overrepresentation analyses were performed for FNDF-LC_{50} and SCFA-LC_{50} groups independently using the DAVID annotation analysis system (da

Table 3 Functional clustering of gene annotations using the DAVID resource reveals enrichment for genes involved in apoptosis in both treatments gene set (hgf-FNDF-LC_{50} and SCFA-LC50, respectively)

Category	Term	Count	*P* value	FDR
Top cluster for the set of hgm-FNDF-LC_{50} target genes				
GOTERM_BP_FAT	Regulation of apoptosis	37	3.3E−27	5.5E−24
GOTERM_BP_FAT	Regulation of programmed cell death	37	4.6E−27	7.7E−24
GOTERM_BP_FAT	Regulation of cell death	37	5.3E−27	8.8E−24
GOTERM_BP_FAT	Apoptosis	33	4.0E−26	6.7E−23
GOTERM_BP_FAT	Programmed cell death	33	6.4E−26	1.1E−22
GOTERM_BP_FAT	Cell death	33	1.0E−23	1.7E−20
GOTERM_BP_FAT	Death	33	1.2E−23	2.1E−20
SP_PIR_KEYWORDS	Apoptosis	23	1.5E−21	1.8E−18
GOTERM_BP_FAT	Positive regulation of apoptosis	23	3.1E−17	5.2E−14
GOTERM_BP_FAT	Positive regulation of programmed cell death	23	3.6E−17	6.0E−14
GOTERM_BP_FAT	Positive regulation of cell death	23	4.0E−17	6.6E−14
GOTERM_BP_FAT	Induction of apoptosis	19	8.2E−15	1.4E−11
GOTERM_BP_FAT	Induction of programmed cell death	19	8.7E−15	1.4E−11
GOTERM_BP_FAT	Induction of apoptosis by intracellular signals	10	4.0E−12	6.6E−9
Top cluster for the set of SCFA-LC_{50} target genes				
GOTERM_BP_FAT	Regulation of apoptosis	37	3.3E−27	5.5E−24
GOTERM_BP_FAT	Regulation of programmed cell death	37	4.6E−27	7.7E−24
GOTERM_BP_FAT	Regulation of cell death	37	5.3E−27	8.8E−24
GOTERM_BP_FAT	Apoptosis	33	4.0E−26	6.7E−23
GOTERM_BP_FAT	Programmed cell death	33	6.4E−26	1.1E−22
GOTERM_BP_FAT	Cell death	33	1.0E−23	1.7E−20
GOTERM_BP_FAT	Death	33	1.2E−23	2.1E−20
SP_PIR_KEYWORDS	Apoptosis	23	1.5E−21	1.8E−18
GOTERM_BP_FAT	Positive regulation of apoptosis	23	3.1E−17	5.2E−14
GOTERM_BP_FAT	Positive regulation of programmed cell	23	3.6E−17	6.0E−14
GOTERM_BP_FAT	Positive regulation of cell death	23	4.E−17	6.6E−14
GOTERM_BP_FAT	Induction of apoptosis	19	8.2E−15	1.4E−11
GOTERM_BP_FAT	Induction of programmed cell death	19	8.7E−15	1.4E−11
GOTERM_BP_FAT	Induction of apoptosis by intracellular signals	10	4.0E−12	6.6E−9

Huang et al. 2009) and identified term enrichment for biological processes. Of important note, the FNDF-LC_{50} and SCFA-LC_{50} gene sets exhibited similar maximally enriched processes, with "regulation of apoptosis" dominating and statistically significant. In addition to the regular GO overrepresentation analysis, DAVID provides a clustering function that forms sets of overlapping gene categories, highlighting "regulation of apoptosis" followed by "cell cycle" for the two categories (Table 3).

Conclusions

In the present study, we showed that the exposure of HT-29 cells to fermented specific extract from common bean cv Negro 8025 results in the modulating gene expression related with apoptosis and cell cycle; most of the genes analyzed were regulated by SCFA-LC_{50} to a lesser extent, compared to the hgm-FNDF-LC_{50}, supporting previous results on cell behavior, probably due to the participation of other compounds, such as phenolic fatty acids derivatives and biopetides. This suggests that a variety of compounds from the fermentation of the NDF of foods are more effective in controlling cell cycle and apoptosis in CRC cells than bioactive compounds separately. As a whole, we demonstrate that common bean (*P. vulgaris* L.) cultivar Negro 8025 has an inhibiting effect against CRC cells by evaluating its human gut fermented NDF and its modulation on genes involved in p53-related signaling pathways, such as the regulation of apoptosis and cell cycle in human colon adenocarcinoma HT-29 cells. Until our knowledge, it is the first time where the human gut

fermented NDF of common bean is compared with their corresponded synthetic SCFA mixture, mimicking their physiological produced concentrations during human gut microbiota fermentation, some of the most studied compounds on colon cancer protection, on the modulation of gene expression by array approach.

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Conflict of interest None of the authors have a conflict of interest. This article does not contain any studies with human or animal subjects performed by the any of the authors.

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