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RESEARCH PAPER

Effect of rosemary polyphenols on human colon cancer cells: transcriptomic profiling and functional enrichment analysis

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Abstract In this work, the effect of rosemary extracts rich on polyphenols obtained using pressurized fluids was investigated on the gene expression of human SW480 and HT29 colon cancer cells. The application of transcriptomic profiling and functional enrichment analysis was done via two computational approaches, Ingenuity Pathway Analysis and Gene Set Enrichment Analysis. These two approaches were used for functional enrichment analysis as a previous step for a reliable interpretation of the data obtained from microarray analysis. Reverse transcription quantitative-PCR was used to confirm relative changes in mRNA levels of selected genes from microarrays. The selection of genes was based on their expression change, adjusted p value, and known biological function. According to genome-wide transcriptomics analysis, rosemary polyphenols altered the expression of ~4 % of the genes covered by the Affymetrix Human Gene 1.0ST chip in both colon cancer cells. However, only ~18 % of the differentially expressed genes were common to both cell lines, indicating markedly different expression profiles in response to the treatment. Differences in induction of G2/M arrest observed by rosemary polyphenols in the two colon adenocarcinoma cell lines suggest that the extract may be differentially effective against tumors with specific mutational pattern. From our results, it is also concluded that rosemary polyphenols induced a low degree of apoptosis indicating that other multiple signaling pathways may contribute to colon cancer cell death.

Keywords Colon cancer · Dietary polyphenols · Microarray · Nutrigenomics · Transcriptomics

Introduction

Dietary polyphenols are currently receiving considerable attention for their presumed role in the prevention of various degenerative diseases such as cancers and cardiovascular diseases (Araújo et al. 2011). The biological activities of rosemary (*Rosmarinus officinalis*) polyphenols have been investigated by several research groups (Herrero et al. 2010). Carnosol, one of the main polyphenols in rosemary, poses strong antioxidant and chemopreventive activities. This diterpene has demonstrated anti-inflammatory and anticancer activities on prostate, skin, breast, leukemia, and colon cancer (Johnson 2011). Another rosemary diterpene, carnosic acid, has proven antiproliferative activity in colon cancer cells (Visanji et al. 2006), anti-inflammatory properties in neurons (Satoh et al. 2008), and potential to promote differentiation of leukemia cells (Danilenko et al. 2001).

During the last years, owing to the extensive optimization and standardization, gene expression microarray has become a leading analytical technology in Nutrigenomics research and is expected to play also a crucial role in the

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emerging Foodomics field (Herrero et al. 2012) for the investigation of the interactions between nutrients and other bioactive food compounds and genes (García-Cañas et al. 2010). High-density microarrays are widely used for comprehensive gene expression studies in part due to their great transcriptome coverage. After microarray data preprocessing, statistical test is usually carried out in order to detect significant changes in the expression of every gene between repeated measurements in two or more conditions (groups). This multiple testing procedure leads to an increased probability of observing false positives, which grows with the number of statistical tests performed. To alleviate the multiple testing problems, methods for correcting the significance level of individual tests should be applied.

The biological interpretation of microarray data is very challenging. In the conventional approach for analysis of microarray data, only the top few individual genes that are differentially expressed between two conditions (groups) are reviewed. Although such individual genes may demonstrate to be relevant in a given biological context, it is increasingly questioned whether large fold changes in individual genes will have more biological relevance than smaller but coordinated fold changes in a set of genes encoding proteins involved in a particular pathway. Over the last years, the use of biological knowledge accumulated in public databases by means of bioinformatics allows to systematically analyze large gene lists in an attempt to assemble a summary of the most enriched and significant biological aspects (Waagmeester et al. 2008). The principle behind enrichment analysis is that if a certain biological process is occurring in a given study, the co-functioning genes involved should have a higher (enriched) potential to be selected as a relevant group by high-throughput screening technologies. This approach increases the probability for researchers to identify the correct biological processes most pertinent to the biological mechanism under study (Huang et al. 2009). Thus, a variety of high-throughput enrichment tools (e.g., DAVID, IPA, MetaCore, Onto-Express, FatiGO, GOminer, EASE, ProfCom, etc.) have been developed in the last years to assist microarray end user to understand the biological mechanisms behind the large set of regulated genes. Many of these bioinformatics resources systematically map the list of interesting (differentially expressed) genes to the associated biological annotation terms of the Gene Ontology (GO) database (www.geneontology.org) and then statistically examine the enrichment of gene members for each of the terms by comparing them to a reference gene set (whole genome or genes included in the chip). This allows identification of the main biological processes associated with the list of differentially expressed genes (DEGs) obtained from microarray analysis. It is obvious that the application of different cutoff thresholds or criteria for the identification of DEGs will alter the gene profile of the resulting list, which in turn, might affect the results obtained from further enrichment analysis. On the other hand, gene set enrichment analysis (GSEA) approach performs enrichment analysis considering all genomic information available from a microarray analysis rather than focusing on individual genes passing a certain significance threshold.

The aim of this work was to conduct genome-wide transcriptomics analysis to investigate the effect of rosemary extracts enriched in polyphenols in two colon cancer cell lines. Two computational approaches, Ingenuity Pathway Analysis (IPA) and GSEA, were used for functional enrichment analysis as a previous step for a reliable data interpretation obtained from microarray analysis.

Materials and methods

Preparation of rosemary extracts

Five rosemary extracts marked as rom1 to rom5 were obtained from dried rosemary leaves using either supercritical fluid extraction (SFE) or pressurized liquid extraction (PLE) as reported by Herrero et al. (2010). The extracts obtained by PLE with a 100 bar pressure for 20 min under the following conditions of solvent and temperature were as follows: rom1, using water at 100 °C; rom2, using ethanol at 150 °C; and rom3, using water at 200 °C. On the other side, the extracts obtained by SFE at 40 °C for 30 min were as follows: rom4, using supercritical CO_2 and 7 % ethanol at 150 bar; rom5, using supercritical CO_2 at 400 bar. Previous chemical characterization studies provided information on the major phenolic constituents in each extract (Herrero et al. 2010). A major phenolic compound in rom1, rom2, and rom3 extracts was rosmarinic acid with concentrations of 14.19, 16.78, and 8.59 μg/mg extract. Rom4 and rom5 were reported to contain high concentrations of carnosol (226.39 and 224.65 μg/mg extract, respectively) and carnosic acid (151.55 and 106.46 μg/mg extract, respectively), being rosmarinic acid undetected. Carnosol and carnosic acid were also present in rom2 extract at concentrations of 104.26 and 66.23 μg/mg extract, respectively. Rom1 and rom3 contained similar concentration of carnosol (~45 μg/mg extract), and carnosic acid was present at concentrations of 0.01, 66.23, and 5.78 μg/mg in rom1, rom2, and rom3, respectively. The extracts have also been reported to show antioxidant activity (EC_{50}) ranging from 5.3 to 10.5 μg/mL (Herrero et al. 2010). Extracts were diluted at the indicated concentrations using ethanol and their content in polyphenols determined as gallic acid equivalents (GAE), expressed as mg gallic acid/g extract.

Total concentrations of polyphenols (given as μM) in the extracts were then calculated using the molecular weight of gallic acid as average value.

Cells and cell culture

Colon adenocarcinoma HT29 and SW480 cells obtained from IMIM (Institut Municipal d'Investigació Médica, Barcelona, Spain) and ATCC (American Type Culture Collection, LGC Promochem, UK), respectively, were grown in DMEM supplemented with 5 % heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 U/mL penicillin G, and 50 μg/mL streptomycin, at 37 °C in humidified atmosphere and 5 % CO_2. Cells were plated at a density of 10,000 cells/cm^2 in 60-mm-diameter culture plates and permitted to adhere overnight at 37 °C. When cells reach 80 % confluence, they were trypsinized (1 mL/25 cm^2), neutralized with culture medium at 1:5 ratio (trypsin/medium) and pelleted for further analysis.

Antiproliferative activity assays

To study the effect of rosemary extracts on the proliferation of HT29 and SW480 lines, cells were seeded onto 60-mm-diameter culture plates at 10,000 cells/cm^2, permitted to adhere overnight at 37 °C, and exposed to different rosemary extracts containing 0–10 μM total polyphenols for 0–72 h depending on the experiment. After incubation with the rosemary extracts for the indicated time in each case, cell proliferation was estimated by the MTT assay as follows: the MTT reagent was added and incubated for 3 h at 37 °C in humidified 5 % CO_2/air atmosphere. After the incubation, the media were aspirated and 200 μL of DMSO was added to each well to dissolve the formazan product by shaking for 30 min. Then, the absorbance at 570 nm was measured in a microplate reader (Anthos 2001 Labtec Instruments GmbH, Wals, Austria).

Cell cycle study

Cell cycle analysis was carried out essentially as previously described (Carrasco-García et al. 2011). Briefly, for cell cycle distribution of DNA content, control and cells treated with the different phenolics were trypsinized, washed with PBS, and fixed with 75 % cold ethanol at −20 °C for at least 1 h. Then, cells were incubated with 0.5 Triton X-100 and 25 μg/mL RNase A in PBS, stained with 25 ng/mL propidium iodide, incubated for 30 min in the dark, and analyzed using an Epics XL flow cytometer equipped with a 0.75 W argon laser set at 488 nm (Beckman Coulter Co., Miami, FL). From the cellular distribution pattern of DNA, the apoptosis induced by treatment of the cells with the phenolic extracts was measured by determining the amount of apoptotic cells in the sub-G1 peak by flow cytometry. Flow cytometry data analysis was made upon gating the cells to eliminate dead cells and debris. A total of 105 cells were measured during each sample analysis.

Microarray analysis

Triplicate samples for each colon cancer cell type, treatment condition, and their respective controls were collected for gene expression microarray analyses. Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Spain) according to manufacturer protocol. The quality of the isolated RNA was determined with a NanoDrop ND1000 (Thermo, Spain) and a Bioanalyzer 2100 (Agilent, Spain). Only those samples with A260/A280 ratio between 1.8 and 2.1 and a 28S/18S ratio within 1.5–2 were further processed. Each triplicate RNA sample was prepared and hybridized onto a separate microarray (Servicio de Genómica, Parque Científico de Madrid, Spain). A starting amount of 200 ng of total RNA was used for double-stranded cDNA synthesis and generation of biotin-labeled cRNA, following the manufacturer protocol (Affymetrix, UK) prior to hybridization onto Human Gene 1.0ST chips (Affymetrix). Generated CEL files were subjected to quality assessment using Expression Console™ (Affymetrix). In this process, summary statistics was computed for each array and then compared across the arrays. Then, CEL files were processed using the Robust Multi-Array (RMA) normalization in the BioConductor package affy for R (Gentleman et al. 2004). Significance analysis was performed using the BioConductor package limma (Linear Models for Microarray Data; Smyth 2005). Limma provides a number of summary statistics useful to select DEGs. Moderated *t* statistics was used for significance analysis of each probe set and each contrast. Then, Benjamini and Hochberg's (1995) method was applied for multiple testing correction. This procedure allows the control or estimation of the false discovery rate (FDR) in a particular data set. To identify the statistically most significant changes in gene expression, microarray data were subjected to gene filtering based on a combination of *M* value cutoff, which represents a $\log_2$-fold change between the two experimental conditions (treatment with polyphenols vs. control), and the statistical significance (FDR applied on moderated *t* statistics). In this study, DEGs were identified based on 0.7 as *M* value cutoff that corresponds to expression ratios (fold change) $\geq$1.6 for up-regulated and $\leq$0.6 for down-regulated genes; and the statistical filter was established at 5 % FDR (adjusted *p* value <0.05).

Reverse transcription quantitative-PCR (RT-qPCR) validation of gene expression

RT-qPCR was used to confirm relative changes in mRNA levels of selected genes from microarray data sets. The selection of genes was based on their degree of expression change, adjusted p value, and/or known biological function. Starting amounts of 0.5 μg of total RNA isolated from cells were reverse transcribed in a volume of 20 μL using Transcriptor First Strand cDNA Synthesis kit with oligo(dT) primers (Roche Diagnostics, Barcelona, Spain). Each real-time quantitative PCR was performed on 0.5 μL aliquots of diluted (1:10) cDNA solutions using LightCycler® 480 Real-Time PCR (Roche Diagnostics) and LightCycler® 480 Probes Master kit. Human Universal Probe Library probes and target-specific PCR primers were selected using the Probe Finder assay design software (Roche Diagnostics, http://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp). Primers were designed to span exon–exon junctions and to have melting temperature values close to 60 °C. The designed primers were then checked with Oligo Analyzer 3.1 software (Integrated DNA Technologies, http://eu.idtdna.com/analyzer/Applications/OligoAnalyzer) to predict possible secondary structures, heterodimers and homodimers, and to redesign the primers if needed. The primers were purchased from Fisher Scientific (Alcobendas, Spain). Supplementary Table S1 summarizes the primers and probes used in this study. Two technical replicates were performed for each sample in a 96-well format plate. On each plate, four endogenous control genes (*GAPDH*, *B2M*, *IPO8*, and *PPIA*) and no-template-controls (NTC) were also performed in duplicate. Cycle-threshold (Ct) values were calculated using second derivative maximum method, and the amplification efficiency (E) of each system was calculated by the following formula: $E = 10^{-1}/\text{slope}$. All primers utilized displayed PCR efficiencies higher than 90 %. Using the Relative Expression Software Tool (REST, Pfaffl et al. 2002), the relative expression of selected genes, calculated using efficiency correction option, in treated cells was compared to that of control cells. The randomization test method, as a part of the REST software, was used to assess statistical significance of up- or down-regulation of target genes after normalization to the four reference genes. Spearman's rho test was used to perform correlation analysis between the microarray and qPCR results for the selected gene set.

Functional enrichment and pathway analysis

In this work, two computational tools were used for functional enrichment analysis as a previous step for a reliable data interpretation obtained from microarray analysis. The bioinformatics tool Ingenuity Pathway Analysis (IPA, Ingenuity Systems, USA) was used in order to interpret the gene expression data in the context of biological processes and pathways. To this aim, the *Core Analysis* function included in IPA was applied to analyze the lists of DEGs identified in microarray analysis. Up- and down-regulated identifiers were defined as value parameters for the analysis. Based on the list of identifiers, IPA performs functional enrichment analysis in order to identify the biological processes and functions over-represented in a given list of genes. Significance of the molecular and cellular functions, as well as the signaling pathways was tested by the Fisher's exact test p value.

As an alternative approach to identify biological processes and functions that are modulated upon exposure to rosemary polyphenols, exploratory functional analysis was also performed with Gene Set Enrichment Analysis (GSEA, Subramanian et al. 2005). In order to implement the GSEA algorithm, RMA-normalized microarray data were uploaded into GSEA v2.0 software (http://www.broadinstitute.org/gsea/index.jsp). Then, for the analysis of each cell line, the GSEA algorithm ranked all microarray genes according to their expression under each experimental condition (treated and untreated). The resulting ranked score values are therefore a function of the correlation between a gene signal intensity, the experimental conditions in question, that is, treatment or control and all other genes in the data set. This enrichment strategy uses a priori defined gene sets to determine whether the members of a given gene set are randomly distributed throughout the ranking, or to the contrary, are primarily found at the top (induced gene expression) or bottom (repressed gene expression) of the rank list of genes. To do this, enrichment scores (ES) are calculated in order to determine the extent to which individual genes from a gene set are represented at the extremes of the ranked gene list. A null distribution of ES is subsequently generated by permutation filtering to evaluate the statistical significance of the observed ES values. After this procedure has been repeated for each gene set, the ES are normalized (NES) to account for differences in gene set size. Then, the false discovery rate (FDR %) is calculated relative to the NES values to determine the false-positive rate. In this work, significant p values and FDR were defined as <0.05 and 25 %, respectively. GSEA analyses were conducted to compare treated versus control cells, for both colon cancer cell lines, SW480 and HT29, using the C5.BP catalog of gene sets from Molecular Signatures Database v3.0 (MSigDB, http://www.broadinstitute.org/gsea/msigdb/index.jsp) containing 850 gene sets consisting of genes annotated by the Gene Ontology (http://www.geneontology.org) biological process terms. Based on the aforementioned cutoff values, leading-edge analyses were

performed on the list of over-represented gene sets (GO terms) to identify leading-edge subsets of genes, defined as the core of a gene set that accounts for the enrichment signal.

Results

Colorectal cancer is known to be strongly linked to dietary factors. Two colon carcinoma cell lines were selected in this study to investigate the effects of rosemary polyphenols on cell viability, cell cycle, and gene expression. SW480 and HT29 cell lines were chosen because they represent different genetic abnormalities of human colon cancer. Both lines contain truncated or mutant adenomatous polyposis coli (*APC*) gene, but differ in mutated *P53* and *RAS* gene, namely, HT29 cells have a mutated *P53* gene (Arg to His-273) but a wild-type *RAS* gene, whereas SW480 cells contain mutated *P53* (Arg to His-273 and Pro to Ser-309) and mutated *RAS* (Val to Glu-12) genes.

Effects of rosemary extracts on cell proliferation

To determine the effective concentration range, dose effect of rosemary polyphenols on cell proliferation in HT29 and SW480 cell lines was evaluated after exposure of the cells to different concentrations of each extract by MTT assay. Rosemary extracts concentrations of 0.1 and 1 μM of polyphenols did not exert significant effect on colon cancer cell proliferation after 72-h incubation with any of the tested extracts (data not shown). Incubation with higher concentrations (10 μM polyphenols) reduced cell proliferation in HT29 and SW480 cells after treatment for 48 h with rom2, rom4, and rom5 rosemary extracts, while rom1 and rom3 extracts did not exert appreciable effect. In general, SW480 cell line was notably more sensitive to the antiproliferative effect of rosemary extracts, especially to rom4 and rom5 extracts, which reduced cell viability to 0 and 18.47 %, respectively, after incubation for 48 h. Therefore, 10 μM total polyphenols solutions from rosemary extracts rom2, rom4, and rom5 were selected for the subsequent experiments.

Cell cycle analysis

To determine whether the antiproliferative effect of rosemary extracts on SW480 and HT29 cells was accompanied by cytotoxicity, distribution of cellular DNA of the cells upon incubation with the extracts and further staining with propidium iodide was analyzed by flow cytometry (FCM). Because HT29 cells were more refractory than SW480 cells to the antiproliferative activity of rosemary extracts, distribution of cellular DNA in the former was done at larger incubation times (72 h) than in the case of SW480 cells (24 and 48 h). Cell cycle analysis showed that rom2, rom4, and rom5 tend to induce a modest apoptotic effect indicated by the small sub-G1 cell population with respect to control cells (data not shown). G2/M arrest was concomitant to a decrease on G1 in SW480 cells treated with rom4 and rom5 extracts, as well as in HT29 incubated with rom4. This effect was especially apparent in the case of SW480 cells incubated with rom4 for 48 h, indicating that the G2/M arrest was sustained in time. Therefore, rom4 extract was selected and its antiproliferative effect against colon cancer cells investigated via transcriptomic analysis.

Microarray analysis and validation of selected targets by RT-qPCR

Gene expression microarray analyses were carried out in order to investigate at transcriptomic level the effect of rom4 extract on both colon cancer cell lines. For this analysis, total RNA was isolated from triplicate cultures of cells incubated with rom4 extract for 24 h (SW480 cells) and 72 h (HT29 cells), together with their respective untreated controls. After microarray data preprocessing, empirical Bayes linear models for statistical analysis (Limma) were implemented in the microarray analysis pipeline to identify DEGs in response to the treatment. Predefined M value ($M = 0.7$) and FDR of 5 % (adjusted p value <0.05) were applied to identify DEGs in response to the treatment with rom4 extract. According to these criteria, within the full list of 28,132 genes represented in the microarray, 1,250 and 1,308 genes were found in SW480 and HT29, respectively, to be differentially expressed in response to the treatment with rom4 extract (Supplementary material, Table S2 and S3). Among these DEGs, the expression of 234 genes ($\sim$18 % of DEGs) was commonly altered in both cell lines, while the expression of 23 genes ($\sim$2 % of DEGs) was altered in both cell lines but with opposite direction of change (up-regulated vs. down-regulated).

Among the lists of DEGs, we selected for RT-qPCR validation a set of genes induced in both lines with remarkable significance levels and expression ratios. Based on this criterion, *HMOX1*, *OSGIN1*, *DUSP1,* and *ARRDC3* genes were selected for validation. We included an additional gene, *MUC1*, that showed different expression pattern in both cell lines and whose expression is associated with poor prognosis in colon cancer (Finn et al. 2011). In this study, *GAPDH* gene and other three reference genes (*IPO8*, *PPIA,* and *B2M*), that have been reported to provide good normalization of RT-qPCR data in colon cancer studies (Sorby et al. 2010), were used for data normalization of selected genes. As shown in Table 1, changes in the expression ratio of all selected genes, except *MUC1* in

Table 1 Comparison of gene expression ratios in response to rom4 treatment as determined by microarray analysis and RT-qPCR in colon cancer cell lines SW480 and HT29

Gene symbol	SW480				HT29			
	Microarray		qPCR		Microarray		qPCR	
	FC[a]	*p* value[b]	FC[a]	*p* value[c]	FC[a]	*p* value[b]	FC[a]	*p* value[c]
HMOX1	24.6	0.0000001	141.6	0.030	3.0	0.0003202	4.0	<0.001
OSGIN1	3.6	0.0003437	13.3	0.035	3.1	0.0014385	5.6	<0.001
DUSP1	11.4	0.0000004	39.5	0.035	2.5	0.0006308	3.0	<0.001
ARRDC3	7.7	0.0000013	15.2	<0.001	5.0	0.0000195	4.6	<0.001
MUC1	1.0	0.7230161	0.7	0.624	0.4	0.0035951	0.3	<0.001

[a] Fold change (expression ratio)

[b] Adjusted *p* value (FDR)

[c] Statistical significance calculated by REST

SW480, were provided by REST with high statistical significance (p value <0.05). The change in the gene expression of *MUC1* was not statistically significant in the microarray analysis of SW480. Therefore, gene expression ratios obtained with RT-qPCR confirm the results obtained by microarray. Significant correlation of 0.964 was observed between data sets obtained by both technologies (Spearman's rho, $p < 0.0000007$).

Functional enrichment analysis and pathway analysis using IPA

In order to identify the biological processes that might be altered in response to rosemary polyphenols, functional enrichment analysis was performed on microarray data sets using different bioinformatics approaches. Enrichment analysis using IPA software was first performed. Lists of identified DEGs obtained from microarray analyses were imported into IPA software. In these analyses, 1,103 and 1,045 genes from SW480 and HT29 microarray data, respectively, were eligible for biological function and pathway analysis. Functional analysis identified significant (Fisher's exact test p value <0.05) over-represented molecular and cellular functions in the imported data sets that were associated to *cell death*, *cellular development*, *cellular growth and proliferation*, and *cell cycle*. Further enrichment analysis on defined (canonical) pathways of IPA Knowledge Base provided significant over-represented pathways across the entire lists of DEGs (Fig. 1). Retinoid X receptor α (*RXRα*) function was identified in three closely related canonical pathways, namely, *LPS/IL-1-mediated inhibition of RXR function*, *xenobiotic metabolism signaling*, and *PXR/RXR activation* in the analysis of treated HT29 cells, and also, in *VDR/RXR activation* pathway identified in the analysis of treated SW480 cells. IPA revealed altered expression of a remarkable number of genes associated to xenobiotic metabolism function as one of the main effects derived from the treatment of HT29 with rom4 extract. In this cell type, examination of *LPS/IL-1-mediated inhibition of RXR function* signaling pathway evidenced strong transcriptional down-regulation of a number of genes targeted by several nuclear receptors, including, *RXRα*, pregnane X receptor (*PXR*), constitutive androstane receptor (*CAR*), and peroxisome proliferator–activated receptor (*PPAR*) (see Fig. 2). Genes encoding phase I metabolizing enzymes, namely, *FMO*, *SOD3*, *CYP3A7*, *CYP2B6*, *CAT,* and several members of *ALDH* gene family, were down-regulated in treated HT29 cells. Also, mRNA levels of the phase II metabolizing enzymes *PAPSS2* and *SULT*, and lipid metabolism–related genes such as cytosolic and mitochondrial *HMGCS* genes were decreased suggesting modulation of xenobiotic and lipid metabolism. On the other side, induction of phase III transporters, *MRP2* and *ABCB9*, was observed. In this canonical signaling pathway, over-expression of two members of IL-1 receptor (*IL1RAP* and *IL1RAPL1*) was coincident with induction of pro-inflammatory cytokine *IL-1β* and *TGFB2*. Coincident with this, IL-1 receptor antagonist (*IL1RN*) was notably over-expressed, whereas TNF receptor (*TNFRSF11B*) and *TLR4* mRNA levels were decreased. In treated SW480 cells, some differences were found in the expression pattern of genes involved in this pathway, namely, the expression of cytokine *IL-1α* was induced, while the mRNA level of *TGFB2* was remarkably decreased. Also, no significant expression changes were detected in the mRNA levels of genes involved in cytochrome P450-mediated phase I metabolism of xenobiotics in this cell line (data not shown). On the other hand, similar to the results obtained in treated HT29 cells, expression of phase III transporters (MRP2 and MDR1) was induced, and it was coincident with down-regulation of gene expression associated with fatty acid and lipid metabolism (*FABP*, *FATP,* and *ACS*). Further examination of *Xenobiotic metabolism* signaling pathway obtained from enrichment

analysis of HT29 transcriptomics data allowed identification of aryl hydrocarbon receptor (*AHR*)-mediated transcriptional induction of phase I metabolizing genes, *CYP1A1* and *CYP1B1*.

In addition to the aforementioned *AHR*/*RXR*/*CAR*/*PXR*-regulated phase I and II enzymes, xenobiotic metabolism signaling may also be regulated by nuclear factor (erythroid-derived 2)-like 2 (*NRF2*). The analysis of *Xenobiotic metabolism* signaling pathway indicated over-expression of phase II detoxifying and antioxidant genes, *GCLC* and *HMOX1* and down-regulation of *UGT* in treated HT29 cells (data not shown). Interestingly, analysis of SW480 suggested that rosemary extract also exerted enhanced antioxidant and xenobiotic detoxifying effects in this cell type through the modulation of *NRF2* function, as it is observed in the enriched canonical pathway *NRF2-mediated oxidative stress response* identified with IPA (Fig. 3). Examination of the downstream target genes of *NRF2* suggested transcriptional *NRF2*-mediated induction of several phase II detoxifying and antioxidant genes, including *HMOX1* (heme oxygenase-1), *TXNRD1* (cytoplasmic thioredoxin reductase-1), and *GCLM* (glutamate-cysteine ligase, modifier subunit) in both colon cancer cell lines. The expression of the antioxidant and tumor suppressor gene *OSGIN1* was also induced in both cell lines. Although IPA did not include this gene in *NRF2*-mediated response to oxidative stress, the expression of *OSGIN1* is known to be regulated via *NRF2* pathway and to follow similar expression pattern to that of *HMOX1* and *GCLM* in response to oxidative signals. In addition to these genes, *FTH1* (ferritin heavy chain) and *SOD2* (mitochondrial superoxide dismutase) genes were induced in SW480,

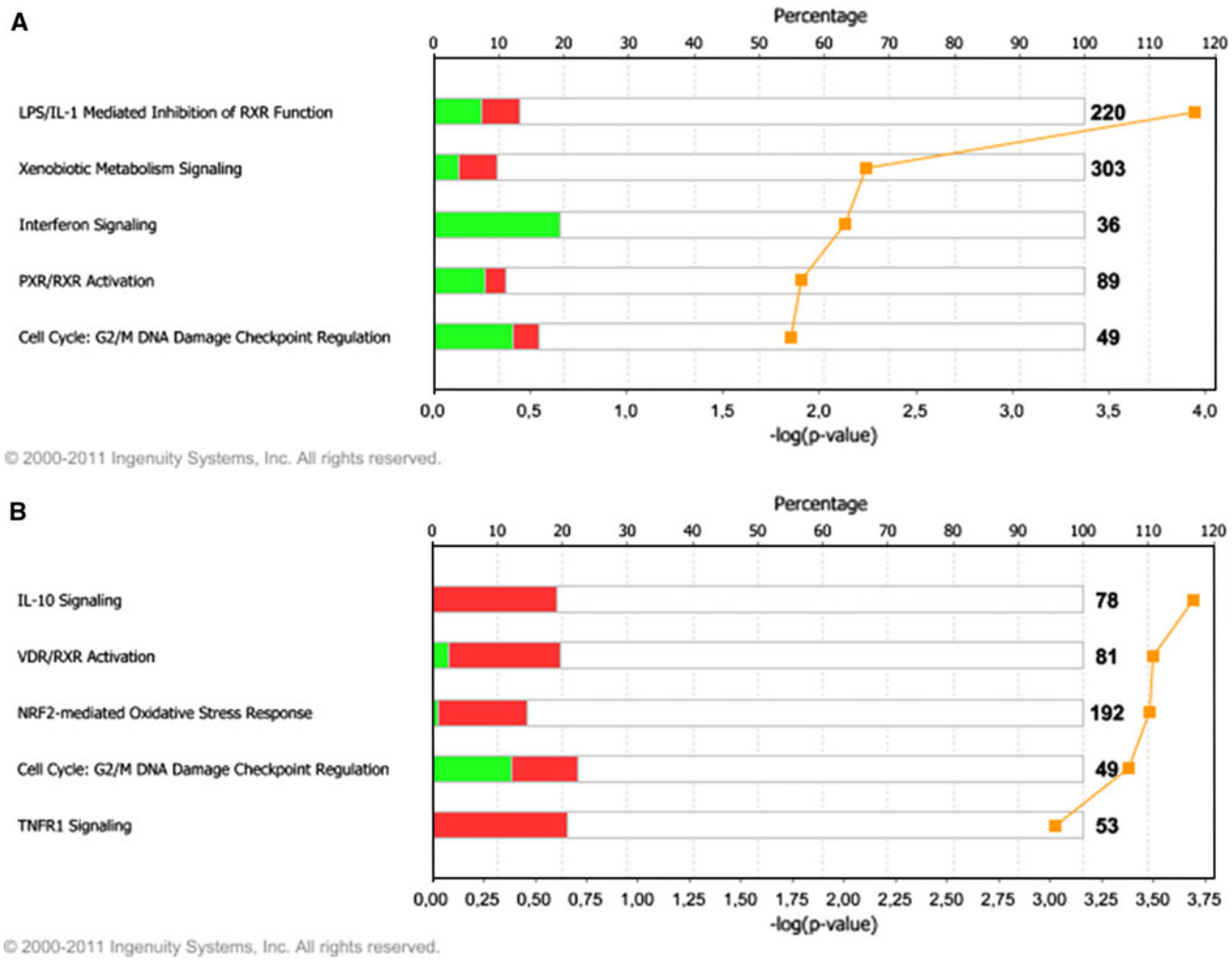


Fig. 1 Canonical pathways significantly modulated by rosemary polyphenols. SW480 (**a**) and HT29 (**b**) colon cancer cells treated for 24 and 72 h, respectively, with rom4 extract. Fisher's exact test was used to calculate a *p* value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance. Stacked histogram displays % of up- and down-regulated genes in a given pathway. *Light* (*green*) *bars* represent the fraction of down-regulated genes. *Dark* (*red*) *bars* represent the fraction of up-regulated genes. Values at the *right* side indicate the number of genes involved in a particular pathway (color figure online)

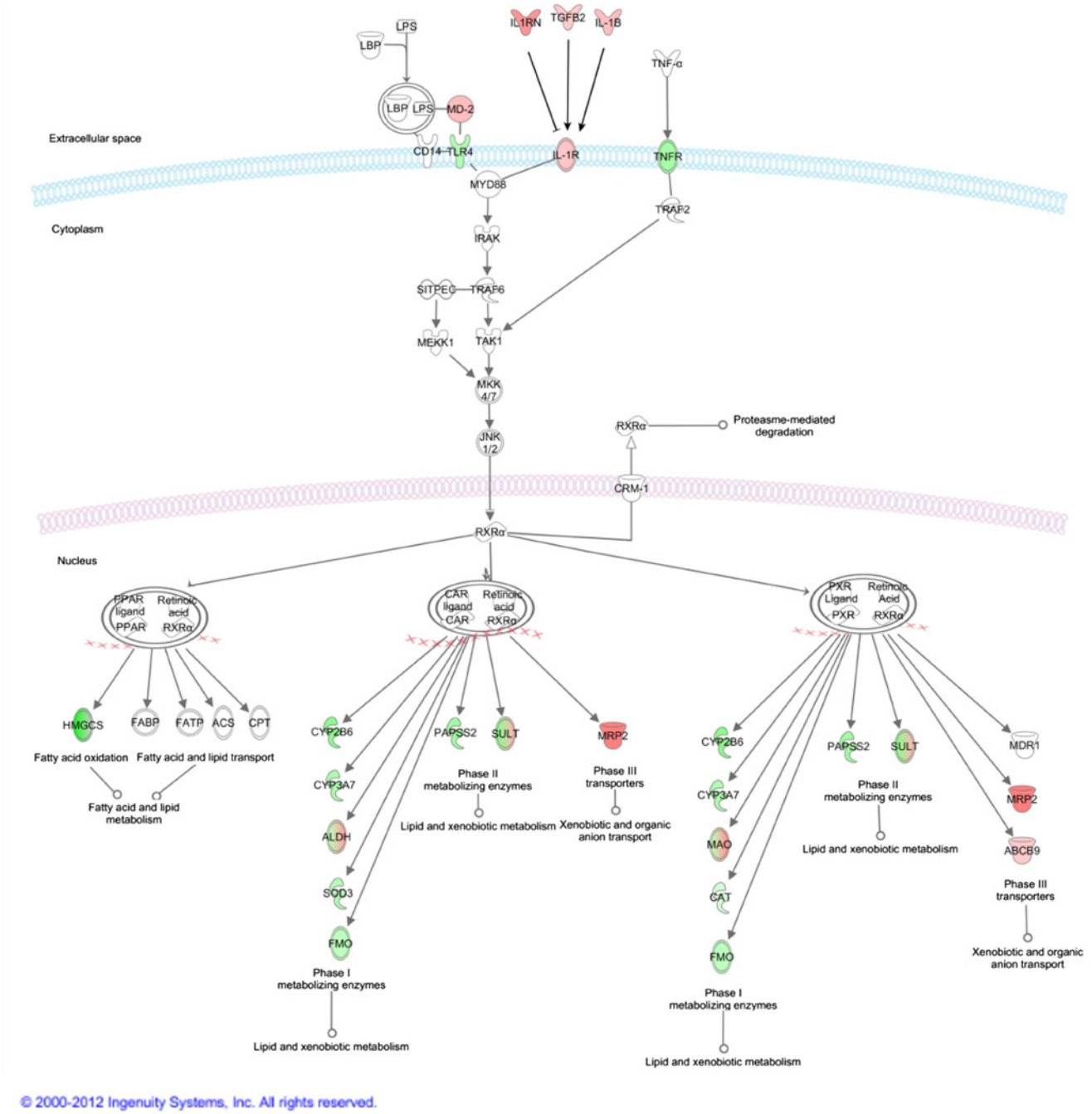


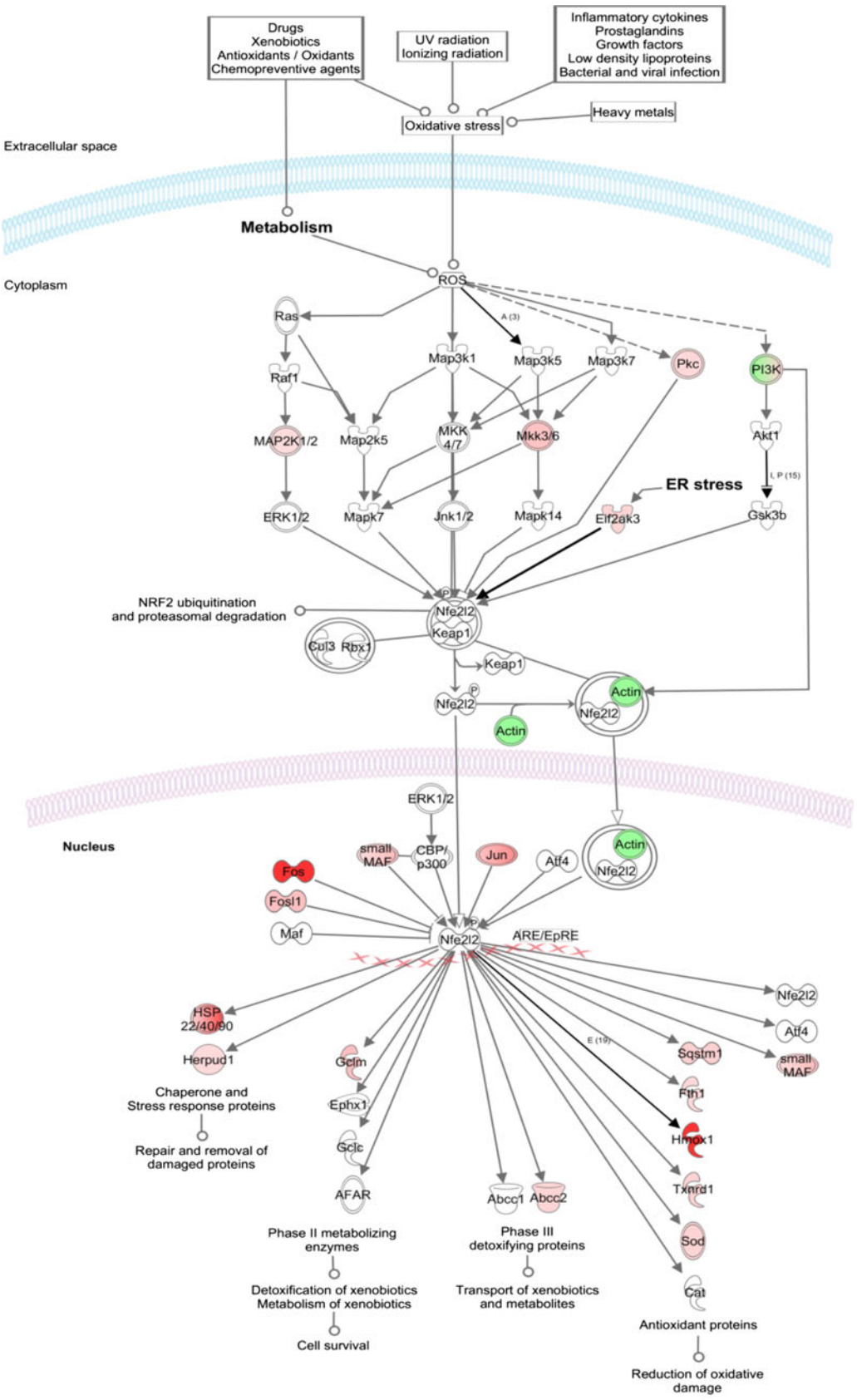
Fig. 2 Canonical pathway representing LPS/IL-1-mediated inhibition of RXR function obtained by IPA from the analysis of HT29 data set. Up- and down-regulated genes are in *red* and *green,* respectively (color figure online)

whereas *GCLC* (glutamate–cysteine ligase, catalytic subunit) and *EPHX1* (epoxide hydrolase 1) genes were up-regulated in HT29. Also, associated with this pathway, the transcript levels of the phase III transporter *ABCC2* were increased in both cell types upon treatment. Observation of transcript levels of upstream signaling molecules of *NRF2* function in SW480 analysis indicated strong induction of genes encoding for protein members of adaptor protein-1 (AP-1), precisely, *C-FOS*, *C-JUN,* and *JUNB*, as well as moderate induction of genes encoding mitogen-activated protein kinases (*MEK1/2*), protein kinase C (*PKC*), and eukaryotic translation initiation factor 2-alpha kinase 3 (*EIF2AK3*), also known as *PERK*. This transmembrane protein kinase has been defined as the central regulator of translational control during unfolded protein response and also represents a linking node between *NRF2* function and endoplasmic reticulum (ER) stress. To expand on our observations in this aspect, we sought to identify up and

downstream molecules mediating ER stress signaling in the transcriptomics data sets using IPA software. As can be observed in Fig. 4, mRNA levels of transcription factors with a known role in ER stress, *XBP1* and *ATF4*, remained unaltered. However, significant induction on the expression of their respective regulated genes was observed. These include the expression of *PPP1R15A* (GADD34) and other ER chaperones (*DNAJB9* and *DNAJB2*). It is worth noting that DNA-damage inducible transcript gene (*DDIT3*) gene expression was notably high in both cell types. Interestingly, the expression of *TRIB3* gene, a stress inducible gene by *DDIT3* and *ATF4*, that recently has been associated with ER stress–dependent apoptosis, was also over-expressed in treated cells. Consistent with the transcriptional activity of *DDIT3*-encoded protein (CHOP, GADD153), its over-expression in SW480 cells was associated with a concomitant increase in the mRNA levels of its inducible pro-apoptotic target genes, *BAK1 (BAK)* and *BCL2L11 (BIM)*. ER resident caspase 4 (*CASP4*) as well as downstream caspase genes, *CASP3*, *CASP7* and *CASP9*, were moderately over-expressed in treated SW480. Other strongly up-regulated genes associated to ER stress transcriptional activation were *DDIT4* gene, an inhibitor of the mTOR survival pathway, and growth differentiation factor 15 (GDF15), whose up-regulation is associated with growth arrest in colon cancer cells. In HT29, the levels of death receptor 5 gene (DR5, *TNFRSF10B*) mRNA were up-regulated by treatment with rosemary extract (data not shown).

According to IPA results, *Cell cycle: G2/M DNA damage checkpoint regulation* pathway was found significant in both cell lines. However, examination of genes regulating this pathway indicated different transcriptomic profiles between cell lines. Despite rosemary extract was able to up-regulate the expression of the *CDKN1A* (p21) and *14-3-3σ* (stratifin) gene and down-regulate *CCNB2* (cyclin B) and *TOPO2* genes in these cells, the expression of other important regulatory genes of G2/M DNA damage checkpoint, such as *CHK1* and *CDC25C,* was selectively down-regulated in HT29. On the other hand, the induction of *GADD45A, GADD45B,* and *GADD45G* was detected in SW480, whereas the mRNA levels of *PRKDC*, *ATM* and *WEE1* genes were decreased in the same line. In SW480 analysis, *Vitamin D receptor (VDR)/RXR activation* signaling pathway was also significant. Examination of this pathway revealed transcriptional activation of *VDR/RXR* heterodimer. Accordingly, the expression of several genes including *KLK6*, *CDKN1A* (p21), *MAD*, *GADD45A*, *OPN*, *CALB1*, *CD14,* and *KLF4* was induced.

Complementary to IPA, GSEA was used to identify groups of genes that shared common biological functions in our microarray data sets. For each group of gene sets (GO annotations), GSEA calculated an ES and evaluated statistical significance in the ES. The normalized enrichment score reflected the degree to which a gene set is over-represented in a given data set. GSEA analysis identified 23 down-regulated gene sets that were significant at 25 % FDR in treated HT29 cells with rom4 extract (Table 2), whereas only 8 down-regulated gene sets fulfill the criteria for significant enrichment in the analysis of treated SW480 cells with the same extract. Among the highest scored functional annotations, a number of down-regulated gene sets common to both cell lines were identified. Most of the gene sets shared by both lines were represented with genes involved in different stages of mitosis, including chromosome segregation and cytokinesis. An example of gene enrichment analysis is shown in Fig. 5a for *Sister chromatid segregation* gene set. The heatmap representation of this subset of genes (Fig. 5b) revealed that most of the genes in this GO term were down-regulated after treatment with the rosemary extract. Leading-edge analysis was subsequently applied in order to identify those gene subsets that account for the enrichment signal in each GO term. Table 3 summarizes the statistically significant enriched gene sets grouped on the basis of the leading-edge gene subsets that they share. Leading-edge subset groupings revealed that 4 and 3 GO terms, related with chromosome segregation, shared 10 and 8 genes in HT29 and SW480, respectively. Also, leading-edge analysis showed that a subset of 8 and 9 down-regulated genes was common to cytokinesis annotations in HT29 and SW480, respectively. On the other hand, two wider subsets consisting of 22 down-regulated genes were part of several GO annotations related with M phase of mitotic cell cycle in the treated HT29 and SW480 transcriptomes, respectively. With regard to other down-regulated functions in HT29, leading-edge analysis revealed other categories related to *DNA repair* and *RNA processing*. Within the leading-edge subsets, we focused on those genes exhibiting enough statistical significance (adjusted p value <0.05) obtained from microarray analyses (bold names in Table 3). Examination of significant genes revealed several common features in the transcriptional response of both cell lines to rom4 treatment. More precisely, important G2/M transition genes such as polo-like kinase 1 (*PLK1*) and its activator aurora A (*AURKA*) were identified as down-regulated in both cell lines. Accompanied with down-regulation of G2/M transition and mitotic entry genes, down-regulation of important genes encoding proteins involved in mitotic checkpoint signaling, such as *BUB1,* was detected in both lines. This down-regulation of mitotic checkpoint function was more prominent in treated HT29 cells, showing decreased transcript levels of other important genes for spindle checkpoint control, namely, *MAD2L1*, *ZWINT,* and *NDC80* (*HEC1*). In both cell lines, genes *ESPL1* (separase) and *CENPE*, associated with sister chromatid separation and chromosome segregation, were



◄ **Fig. 3** Canonical pathway representing NRF2-mediated oxidative stress response obtained by IPA from the analysis of SW480 data set. Up- and down-regulated genes are in *red* and *green,* respectively (color figure online)

down-regulated. The expression of cytokinesis genes, *PCR1* and *RACGAP1,* was also decreased in both treated cell lines.

Discussion

Rosemary constituents have demonstrated different in vitro cytotoxicity effects depending on the cell type, concentration, and time of exposition. For instance, studies with carnosic acid showed that concentrations of 6.25 μg/mL reduced between 13 and 30 % the viability of different types of tumor cells (Yesil-Celiktas et al. 2010). Similar effects have been reported for carnosic acid on viability of five human cancer cell lines (Gigante et al. 2003). To the opposite, concentrations of rosmarinic acid ranging from 6.25 to 50 μg/mL (139 μM) showed proliferative effects rather than cytotoxic activity (Yesil-Celiktas et al. 2010). Carnosol exhibited growth inhibition 50 % (GI_{50}) values ranging from 3.6 to 26 μM in five tumor cells (Guerrero et al. 2006). Also, Visanji et al. (2006) reported IC50 values of 23 μM of carnosol for Caco-2 cells, a value close to the observed (34 μM) in prostate cancer cells after 72 h of treatment (Johnson 2011). In our study, rosemary extracts showed different antiproliferative activity on colon cancer cells. More precisely, carnosol-enriched extracts, rom4 and rom5, showed the strongest effect on the proliferation of both cell lines.

In general, rosmarinic acid–enriched extracts (rom1-3) exerted lower antiproliferative activity. Among these extracts, the highest antiproliferative effect was observed in rom2, the extract containing high concentration of carnosol (104.26 μg/mg) and carnosic acid (66.23 μg/mg). On the other hand, rom1, the extract containing traces (0.01 μg/mg) of carnosic acid and 46.11 μg carnosol/mg, exhibited negligible antiproliferative activity. Our data also indicated that rom4 and rom5 extracts inhibited colon cancer cell growth by inducing G2/M cell cycle arrest after 24 and 72 h of exposition in SW480 and HT29, respectively, the effect being maintained for 48 h in SW480 cells with rom4. A low level of apoptosis was observed indicating coexistence of cytostatic and cytotoxic effects induced by treatment with rom4 and rom5 extracts. Induction of G2/M cell cycle arrest by carnosol-enriched extracts (rom4 and rom5) is in consonance with the observations found in the

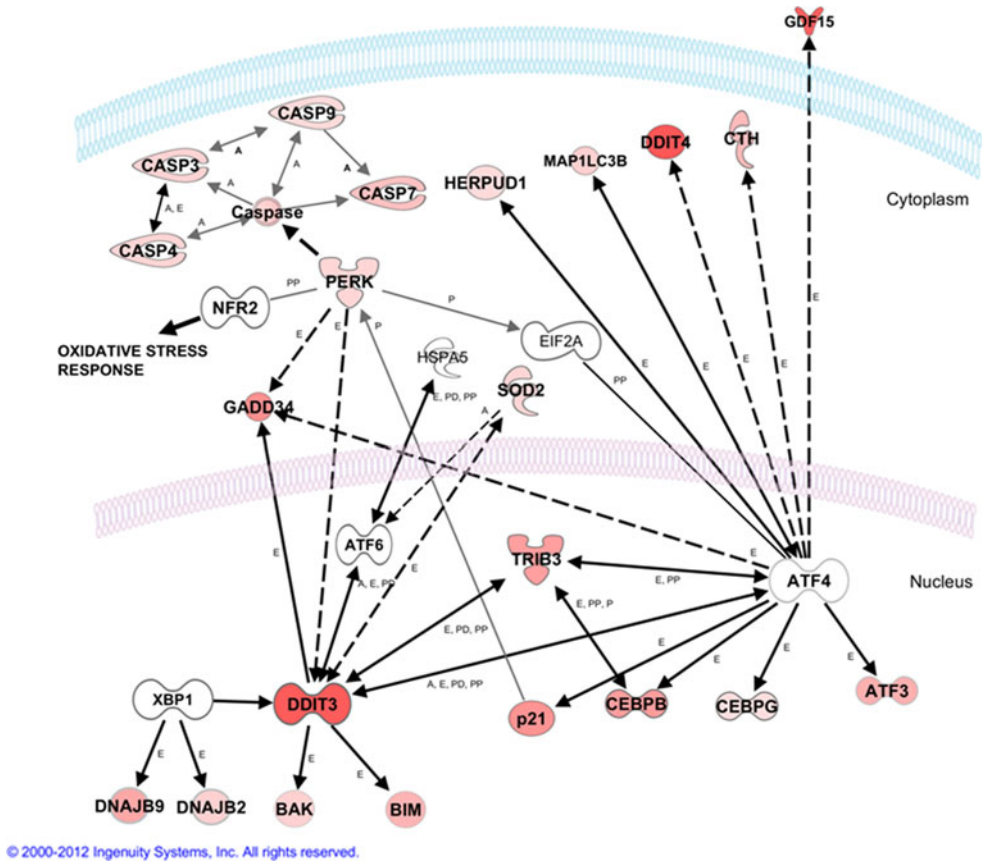


Fig. 4 Gene network obtained from IPA Knowledge Base illustrating direct (*solid line*) and indirect (*dashed line*) interactions between transcriptional regulators and their target inducible genes involved in ER stress modulated by rosemary polyphenols in SW480 cells. The type of interaction is indicated between *brackets*: *E* expression; *A* activation; *P* phosphorylation; *PP* protein–protein interaction; *PD* protein–DNA interaction. Expression type interactions are highlighted by *black arrows*

Table 2 Over-represented down-regulated gene sets microarray transcriptomics data obtained from treatment of colon cancer cells with rom4 extract

Cell line	Name	Size	NES[a]	NOM p val[b]	FDR q val[c]
HT29	Sister chromatid segregation	16	−1.97	0.000	0.044
	Mitotic sister chromatid segregation	15	−2.00	0.000	0.064
	Cell cycle process	176	−1.89	0.000	0.075
	Chromosome segregation	30	−1.84	0.002	0.079
	Cytokinesis	18	−1.85	0.000	0.088
	Mitotic cell cycle	140	−1.78	0.000	0.090
	Cell cycle phase	158	−1.79	0.000	0.092
	Mitosis	75	−1.75	0.000	0.094
	M phase	103	−1.74	0.003	0.096
	M phase of mitotic cell cycle	77	−1.80	0.000	0.097
	Chromosome organization and biogenesis	110	−1.76	0.000	0.098
	DNA repair	107	−1.72	0.000	0.109
	Cell cycle	286	−1.70	0.000	0.120
	Regulation of membrane potential	15	−1.66	0.027	0.138
	mRNA processing	66	−1.67	0.005	0.140
	RNA splicing	76	−1.65	0.000	0.142
	Positive regulation of secretion	17	−1.63	0.014	0.157
	Double strand break repair	20	−1.60	0.022	0.165
	Cell cycle checkpoint	43	−1.61	0.017	0.167
	DNA recombination	41	−1.60	0.020	0.169
	Microtubule cytoskeleton organization and biogenesis	33	−1.58	0.033	0.185
	Response to DNA damage stimulus	138	−1.56	0.006	0.207
	Cell division	20	−1.55	0.043	0.207
SW480	Sister chromatid segregation	16	−1.87	0.003	0.081
	Cell division	20	−1.91	0.005	0.108
	Mitotic sister chromatid segregation	15	−1.81	0.000	0.109
	Cytokinesis	18	−1.77	0.005	0.121
	Chromosome segregation	30	−1.66	0.003	0.199
	Extracellular structure organization and biogenesis	29	−1.62	0.017	0.202
	M phase of mitotic cell cycle	77	−1.62	0.000	0.219
	Mitosis	75	−1.62	0.005	0.244

[a] Normalized enrichment score

[b] Nominal p value

[c] False discovery rate (q value)

literature with carnosol and carnosic acid (Visanji et al. 2006). According to genome-wide transcriptomics analysis, rom4 extract altered the expression of ~4 % of the genes covered by the Affymetrix Human Gene 1.0ST chip in both colon cancer cells. However, only ~18 % of DEGs were common to both cell lines, indicating a markedly different expression profile in one line in comparison with the other in response to the treatment.

Based on IPA results, a noteworthy number of differentially expressed genes were associated with *cellular development*, *cell death*, *cellular growth and proliferation* and *cell cycle*, in both cell lines indicating a clear alteration of similar important biological functions in response to the treatment with rosemary polyphenols. Studies of dietary ingredients, principally phytochemicals, have demonstrated their anticarcinogenic effects as blocking agents. Certain phytochemicals prevent genotoxic carcinogens from forming adducts with DNA, either by inhibiting their activation from pro-carcinogens or by promoting their detoxification and excretion. These actions are mainly controlled by the balance between phase I enzyme activity and phase II detoxifying enzyme activity. Phase I metabolism involves the oxidation, reduction, and hydrolysis of xenobiotics, including drugs, toxins, and carcinogens, mainly by the cytochrome P450 (CYP) enzymes. A common mode for xenobiotic metabolism enzyme regulation is

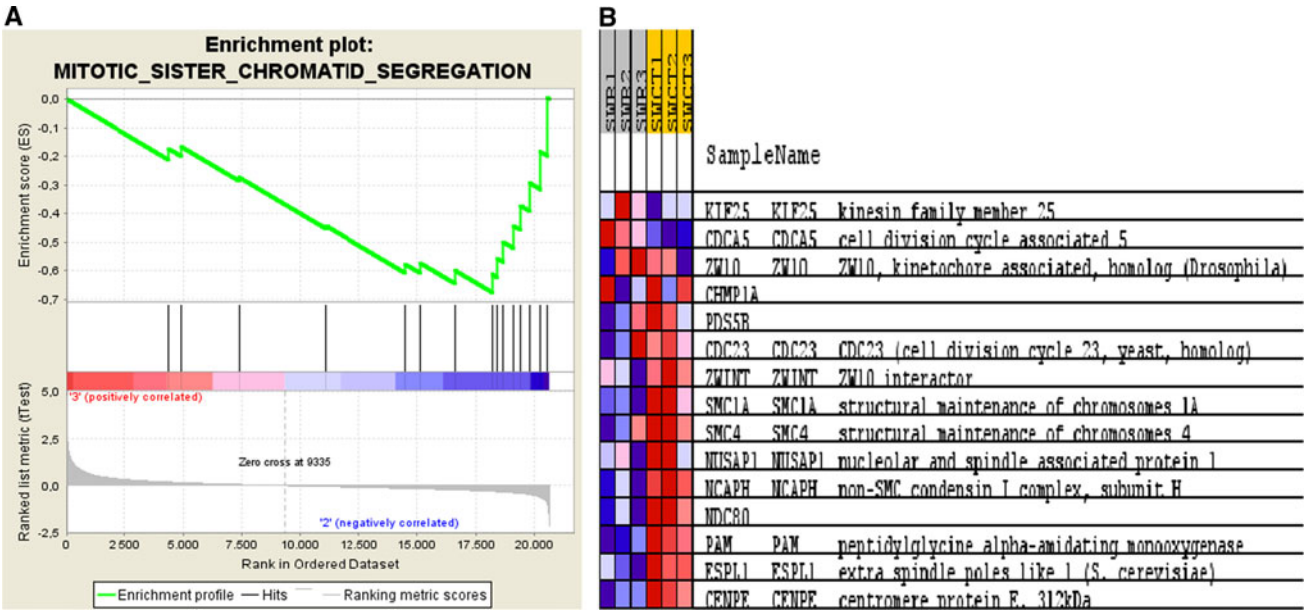


Fig. 5 Example of GSEA enrichment plot for *Mitotic Sister Chromatid Segregation* in SW480. **a** The *x* axis lists all microarray genes ranked based on their association with the comparison treated SW480 versus control. The *black vertical bars* indicate candidate genes in the target gene set. The ES profile (*green* trace) records the cumulative score of the gene ranks from the target gene set. Here, a majority of gene ranks from the candidate gene set are low (i.e., toward the down-regulated genes of the ranking) compared to the rest of genes, and the cumulative ranking score (ES profile) will have a negative peak, suggesting a significant enrichment of this gene set. The significant genes are determined by the position of the peak of the profile. There are 8 candidate genes up to this peak position that are claimed as significant (leading-edge subset). They are plotted in the heatmap shown in **b** (*red* means high expression level, and *blue* means low expression level) (color figure online)

transcriptional control by receptors. Several receptors including *RXR*, *PXR, CAR*, *AHR*, *VDR*, retinoic acid receptor (*RAR*), and glucocorticoid receptor (*GR*) are implicated in xenobiotic metabolism enzymes regulation. According to our transcriptomics data, xenobiotic heterodimer receptors *RXRα/CAR*, *RXRα/PXR,* and *RXRα/PPAR* did not induce transcriptional up-regulation of most of their target genes in treated HT29. To the contrary, a global down-regulated expression of *RXRα*-inducible genes was revealed by IPA analysis. Transcriptional activity of *RXRα* can be decreased by pro-inflammatory cytokine, which induce increased nuclear export of *RXRα*, leading to reduced nuclear *RXRα* levels and decreased nuclear DNA binding. Interestingly, over-expression of members of IL-1 receptor as well as pro-inflammatory cytokine IL1 in both cell types might be responsible for the observed transcriptional inactivation of *RXRα* functions, particularly those affecting phase I metabolizing enzymes and lipid metabolism. However, this effect may be partially counteracted by the up-regulation of the IL-1 receptor antagonist that acts blocking the IL-1 receptor. Activation of the AHR nuclear factor was deduced by the modest over-expression of *CYP1A1* and *CYP1B1* genes observed in treated HT29 cells. The activation of AHR signaling by naturally occurring dietary chemicals including carotenoids, phenolics, and flavonoids has been reported in the literature (Rodeiro et al. 2008). Also, induction of expression of the efflux transporter *MRP2* in both cell lines may be related to a cellular defense response aimed at increasing the excretion of the xenobiotic or their metabolites.

One of the main similarities found on the effect of rom4 extract in both cell lines was associated with the transcriptional activation of typical nuclear factor *NRF2* target genes such as *HMOX1*, *TXNRD1*, *GCLM,* and *OSGIN1* in both colon cancer cell lines; *FTH1* and *SOD2* in SW480; and *GCLC* and *EPHX1* in HT29. These enzymes exert efficient cytoprotection against toxicants through a variety of reactions, promoting elimination or inactivation of toxic reactive species (including potential carcinogens) before they cause damage to critical cellular macromolecules (Martin et al. 2004). In our transcriptomics data sets, the expression level of *HMOX1* gene was remarkably high, especially in treated SW480. RT-qPCR confirmed that *HMOX1* and *OSGIN1* were significantly up-regulated in both cells by rom4. *HMOX1* gene encodes heme oxygenase-1 isozyme that catalyzes the degradation of heme into three products, namely, carbon monoxide (CO), biliverdin, and free iron. Biliverdin is converted to bilirubin by the action of biliverdin reductase, whose expression was also induced in treated SW480. Many reports have demonstrated the potent antioxidant activity of the heme-derived metabolites generated by heme oxygenase-1 catalysis (biliverdin and

Table 3 Leading-edge subsets of genes found in down-regulated GO terms obtained in functional enrichment analysis of microarray data using GSEA

Functional group	HT29		SW480	
	Gene set (GO term)	Leading-edge subsets of genes[a]	Gene set (GO term)	Leading-edge subsets of genes[a]
Cytokinesis	Cell division, cytokinesis	SEPT4, SEPT6, SEPT7, **BRCA2**, **PRC1**, **RACGAP1**, **ANLN**, **NUSAP1**	Cell division, cytokinesis	**INCENP**, **PRC1**, DIAPH2, BRCA2, **RACGAP1**, SEPT6, **MYH10**, BIRC5, NUSAP1
Chromosome segregation	Sister chromatid segregation, Mitotic sister chromatid segregation, chromosome segregation, chromosome organization and biologenesis	**NUSAP1**, CDC23, PDS5B, **ZWINT**, **ESPL1**, **NDC80**, NCAPH, **PAM**, **CENPE**, **SMC4**	Sister chromatid segregation, Mitotic sister chromatid segregation, chromosome segregation	**PAM**, NCAPH, **CENPE**, **ESPL1**, NDC80, SMC4, **SMC1A**, NUSAP1
M phase of mitotic cell cycle	Cell cycle process, cell cycle phase, mitotic cell cycle, mitosis, M phase of mitotic cell, M phase, cell cycle	**TTK**, **KIF2C**, **KIF11**, **CCNA2**, **PML**, **MAD2L1**, **BUB1**, **KNTC1**, **AURKA**, NEK2, **TPX2**, **CIT**, **PLK1**, **KIF15**, **NUSAP1**, **ZWINT**, **ESPL1**, **NDC80**, NCAPH, **PAM**, **CENPE**, **SMC4**	Mitosis, M phase of mitotic cell	**PAM**, NCAPH, **CENPE**, **ESPL1**, NDC80, SMC4, **SMC1A**, BIRC5, **KNTC1**, **TTK**, **AURKA**, **KIF2C**, **BUB1**, **PBRM1**, CCNA2, **KIF11**, **KIF15**, **TPX2**, **SMC3**, MAD2L1, **PLK1**, **CIT**
RNA processing	RNA processing, RNA splicing	PPARGC1A, SFRS6, NONO, SFRS7, SFRS1, SFRS2, SFRS11, CWC15, PRPF31, SRPK2, SF3B3, USP39, SFPQ, TXNL4A, SNRPD1, SMNDC1, EFTUD2, SLU7, SNW1, **TRA2A**, SFRS2IP		
DNA repair	Double strand DNA repair, DNA repair, response to DNA damage stimulus	MRE11A, RAD54B, RAD51, **RAD21**, POLA1		

[a] Bold: genes with adjusted p value <0.05

bilirubin) and the cytoprotective actions of CO on vascular endothelium and nerve cells. Hence, it is now widely accepted that induction of *HMOX1* expression represents an adaptive response that increases cell resistance to oxidative injury. The signaling mechanisms used to activate transcription of *HMOX1* are dependent of the cell type and the inducing agent. For instance, carnosol, a major phenolic compound in rom4 extract, seems to rapidly induce *HMOX1* gene expression through *NRF2* transcription factor in conjunction with the survival signaling pathway represented by phosphatidylinositol 3-kinase (PI3 K)/AKT in mouse PC12 cells (Martin et al. 2004).

Induction of phase II detoxifying and antioxidant enzymes is mediated primarily by the antioxidant or electrophile response elements (ARE/EpRE), which are found in the 5′ upstream promoter region of many of the phase II/antioxidant genes involved in xenobiotic metabolism and oxidative stress response. *NRF2* nuclear transcription factor plays an essential role in ARE-gene expression. In absence of stimuli, *NRF2* element is sequestered in the cytoplasm by binding to *KEAP1* gene product, an actin-binding protein. Under oxidative stress or in the presence of electrophiles, *KEAP1* protein is inactivated by oxidative modification of its cysteine residues, allowing for *NRF2* receptor translocation into the nucleus, where it heterodimerizes with small *MAF* proteins, and binds to AREs. A wide variety of dietary compounds, namely, sulforaphane, curcumin, and caffeic acid phenetyl, that act as potent inducers of ARE-regulated gene expression, have been reported to provide chemopreventive effects (Lee and Surh 2005). Accumulating evidence from in vitro studies with neuronal cells and dietary polyphenols suggests that catechol-type electrophilic compounds such as carnosic acid and carnosol initiate S-alkylation of cysteine thiol of the *KEAP1* protein–inducing protective effects dependent of the cell type by promoting the translocation of *NRF2* from the cytoplasm to the nucleus (Satoh et al. 2008). Induction of ARE-gene expression in a *NRF2*-dependent manner by carnosic acid and carnosol has shown to inhibit adipocyte differentiation in mouse (Takahashi et al. 2009) and to regulate expression of nerve growth factor mediated by *NRF2* activation, conferring protection in glioblastoma cells (Mimura et al. 2011).

It is also interesting to note the observed increase in mRNA levels of *PERK* gene in treated SW480 cells. *PERK*

represents a link node between unfolded protein signaling, in response to ER stress, and *NRF2* function. It has been suggested that *NRF2* activation through *PERK* contributes to the maintenance of glutathione levels, which functions as a buffer against the accumulation of reactive oxygen species during the unfolded protein response caused by ER stress (Cullinan et al. 2003). Treatment of colon cancer cells with rom4 extract was found to induce a number of signature ER stress markers including (*PPP1R15A*, *DDIT3,* and *TRIB3*) suggesting the induction of ER stress. Furthermore, the increased expression of the transcriptional regulator *DDIT3* is often associated with RE stress–induced apoptosis (Rao et al. 2004). This transcription factor is known to regulate the expression of a set of genes including pro-apoptotic genes, including *BAK* and *BIM*, to originate mitochondrial cell death. *TRIB3* protein is an inducible target of *DDIT3* acts as a sensor for ER stress–induced apoptosis. It has been proposed that during mild ER stress, *TRIB3* down-regulates *DDIT3* expression via negative feedback mechanism, allowing the cell to adapt to ER stress. In contrast, induction of *TRIB3* would be more robust during severe or persistent ER stress, promoting apoptosis through inhibition (dephosphorylation) of Akt (Ohoka et al. 2005). This feedback mechanism could facilitate ER stress–mediated apoptosis in severely ER stressed cells that have successfully reached pro-apoptotic threshold levels of *DDIT3* (Zou et al. 2009). Rom4 extract seems to initiate the *DDIT3* pro-apoptotic transcription activity since the expression of its immediate inducible target genes, *BAK* and *BIM,* was also over-expressed in SW480. In this cell line, the increase in the expression of these pro-apoptotic genes was concomitant with the increase in mRNA levels of *CASP4*, which activation has been associated to ER stress (Hitomi et al. 2004). Recent evidences suggest that ER stress may be of importance for the cytotoxic activity of certain phenolic compounds. For instance, treatment with curcumin caused DNA damage in the form of single-strand breaks in HCT116 colon cancer cell and induced oxidative stress. This effect was accompanied by the induction of *DDIT3* gene expression and subsequent apoptosis (Scott and Loo 2004). Huang et al. (2011) have recently reported the pro-apoptotic effects of a phloroglucinol derivative in various human colon cancer cells. The observed effects were mediated by ER stress response through the induction of *DDIT3* and *HSPA5* genes as well as the activation of GSK3α/B, caspases 3, 7, and 9. Similarly, resveratrol has been demonstrated to increase the expression of *DDIT3* and *HSPA5* genes in colon cancer cells (Um et al. 2010). Polyisoprenylated benzophenones inhibited growth of human colon cancer cells by the activation of *DDIT3*, *ATF4,* and *XBP1* genes and inhibition of mTOR cell survival pathway through over-expression of *DDIT4* gene (Protiva et al. 2008). In a recent report, *DDIT3* induction has been also associated with up-regulation of *DR5* and subsequent apoptosis in human colon carcinoma HT29 cells following treatment with the polyphenol rottlerin (Lim et al. 2009). Transcriptomics data suggest that rom4 extract may induce RE stress–dependent apoptosis in colon cancer cells, especially in SW480 cell type. However, this pro-apoptotic effect may be counteracted by other mechanism in light to the low degree of apoptosis observed in vitro. Further investigations are required to elucidate the nature and the underling mechanisms of the cellular stress induced by the extract.

Data revealed strong transcriptional induction of AP-1 members, *C-JUN*, *JUNB,* and *C-FOS* in response to rom4 treatment in SW480 cells. Several works on dietary agents such as sulforaphan, allyl isothiocyanate (AITC), curcumin, (−)-epigallocatechin-3-gallate (EGCG), and resveratrol, with proven chemopreventive and cell growth inhibition activities, modulate AP-1 transcription activation in a cell type- and dose-dependent manner. Excepting EGCG and AITC, most of them induce early and transient transcriptional activation at low-range concentrations (1–50 μM) that is decreased at higher doses (75–100 μM) in colon cancer cellular models (Jeong et al. 2004). AP-1 activity is involved in many diverse cellular processes including apoptosis, proliferation, transformation, and differentiation. Moreover, its activation is often associated with a positive role in cell proliferation. The reasons for the discrepant observation of AP-1 induction and effects observed in vitro after exposure to the mentioned dietary factors are not clear yet.

Vitamin D receptor (VDR)/Retinoid X receptor (RXR) activation pathway was also significant in the treatment of SW480. *VDR* is a member of the nuclear receptor family of transcription factors and exists as a heterodimer with *RXR*. Vitamin D3-bound *VDR-RXR*, along with other co-activator proteins, mediates the transcriptional regulation of a number of genes involved in immune function, tumor suppression, growth regulation, and regulation/metabolism of calcium. Carnosic acid has shown to act synergistically with 1α,25-dihydroxyvitamin D3 by increasing the transcriptional *VDR* levels in HL60 leukemia cells (Danilenko et al. 2001). This effect was directly associated with enhanced differentiation of leukemia cells, suggesting that carnosic acid may also increase binding of VDR/RXRα heterodimers to vitamin D response elements in promoters of VDR-targeted genes. The same effects on cell differentiation have been observed to a certain extent with other antioxidant compounds (i.e., curcumin, silibinin, lycopene, b-carotene). Therefore, it has been suggested that these effects are not dependent on any particular structural similarity, indicating that the antioxidant properties of each molecule may be the common mechanism. Carnosic acid has demonstrated to be capable of protecting cells against

oxidative stress by decreasing the intracellular levels of reactive oxygen species (ROS) concomitant with an increase in total glutathione content. Furthermore, these changes have been suggested to serve as sensing signals of the cellular redox status for the regulation of early response transcription factors, such as AP-1, that may exhibit increased binding capacity to its cognate DNA element in the promoter region of *VDR* gene (Danilenko and Studzinski 2004). In our study, SW480 cells treated with rosemary polyphenols exhibited increased levels of AP-1 and *VDR* transcript. Also, VDR-inducible genes, including *CDKN1A* and *KLK6* (involved in cell differentiation), *MAD* and *GADD45A* (related to growth regulation), and *KLF4* (regulation of cell cycle progression), were over-expressed, whereas *TGFB2* (associated to grown inhibition) was down-regulated. To the contrary, HT29 analysis revealed the transcriptional induction of *VDR*-repressor *NCOR2* coincident with decreased mRNA levels of its repressible target, *IP-10* gene. In this cell line, rom4 also up-regulated the *VDR*-inducible genes *KLF4*, *CDKN1A*, as well as the mediator of tumor suppression *SEMA3B* and *PPARD* (growth regulation).

IPA results provided evidences of alteration of G2/M checkpoint control function by modulation of the expression of important genes, including *CDKN1A* and *14-3-3σ* in both cell lines. Interestingly, rom4 induced the expression of stress-response family of *GADD45* genes in SW480. Recently, induction of *GADD45* gene expression by a secoiridoid-rich phenolic extract form olive oil has been associated with activation of G2/M checkpoint control in breast cancer cells (Oliveras-Ferraros et al. 2011). In addition to IPA results, GSEA provided complementary information regarding the cell cycle progression functions altered in colon cancer cells in response to the rosemary extract treatment. The main benefits of GSEA approach are the reduction in the arbitrary factors in the typical gene selection step that could impact the traditional enrichment analysis; and the use of all the information obtained from microarray analysis by allowing the minimally changing genes, which cannot pass the selection thresholds, to contribute to the enrichment analysis (Huang et al. 2009). GSEA showed that rom4 extract induces changes in a range of cell cycle processes. A significant number of moderately down-regulated genes related with M phase, chromosome segregation and cytokinesis, enriched data sets obtained in gene expression microarray analysis. In our study, *AURKA*, the main activator of *PLK1* protein, was also down-regulated in the treated colon cancer cells in relation to their respective untreated controls, probably affecting centrosome maturation and microtubule dynamics during G2/M transition (Macürek et al. 2008). In recent years, *PLK1* has emerged as an important regulator in cell cycle progression (Barr et al. 2004). *PLK1* is known to play a pivotal role in the regulation of mitotic entry, chromosome segregation, and cytokinesis (Lowery et al. 2005). Owing to the strong association of this kinase with cell proliferation and elevated expression in a variety of tumors, including colon tumors, inhibition of *PLK1* function has been suggested as a potential alternative for cancer therapy (Chopra et al. 2010). Moreover, depletion of *PLK1* is associated with the decrease in cell viability and induction of apoptosis in various cancerous cells (Liu and Erikson 2003). Cancer cells with depleted *PLK1* activity show inability to completely separate the sister chromatids during mitosis. It has been shown that *PLK1*-depleted cancer cells eventually undergo induction of G2/M arrest and apoptosis (Liu and Erikson 2003). Coincident with down-regulation of *PLK1*, several members involved in the mitotic checkpoint function remained also down-regulated in treated cells. Partial loss of checkpoint control by such genes has often been associated to abnormal mitosis (Jeganathan et al. 2007). Among the down-regulated mitotic checkpoint gene members detected in this work, it is noteworthy the down-regulation of *HEC1* gene. Interestingly, *HEC1* has been suggested as a potential target for therapeutic intervention in cancer and hyper-proliferative diseases since its depletion in HeLa cells by siRNA technique has shown to result in mitotic catastrophe, whereas normal checkpoint-proficient cells arrest transiently in response to *HEC1* inhibition (Martin-Lluesma et al. 2002). Dysregulation of sister chromatid and chromosome segregation functions in colon cancer cells after treatment with rom4 extract was evidenced by the altered expression of important genes (*ESPL1* (separase) and *CENPE*). Consistent with the observation that rom4 extract accumulate the colon cancer cells in G2/M phase under assayed conditions in this work, a delay in mitosis entry (arrest in G2/M) and mitosis failure by the coordinated decrease in expression of key genes for mitosis entry and mitotic cell cycle may be expected. Although the support for this hypothesis is derived from the collective down-regulation of several genes involved in G2/M transition, mitosis, and cytokinesis, further investigation is needed in order to elucidate the exact mechanisms underlying the G2/M arrest observed after addition of rosemary extract. Differences in induction of G2/M arrest observed by rom4 in the two colon adenocarcinoma cell lines suggest that the extract may be differentially effective against tumors with specific mutational pattern.

Conclusions

Taken together, our results revealed that rosemary polyphenols markedly affected the viability of the assayed colon cancer cell types through multiple pathways. Thus,

rosemary polyphenols induced chemopreventive effects through transcriptionally activated genes that encode antioxidant phase II enzymes in the colon cancer cell lines. These findings are consistent with the reported *NRF2*-activating effect of carnosic acid and carnosol, two of the major phenolic compounds in the rosemary extract under study. Our data highlight other plausible mechanism by which rosemary polyphenols may also trigger the *NRF2*-mediated induction of detoxification and antioxidant enzymes. This complementary signaling pathway may entail activation of gene products involved ER stress. Evidences of transcriptional activation of pro-apoptotic genes have been collected in this study; however, cell viability experiments with rosemary polyphenols showed modest accumulation of apoptotic cells suggesting other multiple signaling pathways may also contribute. The two computational tools used in the study shed some light on the mechanisms underlying the observed G2/M cell cycle arrest exerted by rosemary polyphenols. IPA helped in providing information on those markedly changed gene expressions on G2/M checkpoint signaling pathway, while GSEA provided information on sets of simultaneously, but moderately altered genes, closely related in G2/M transition, mitosis, and cytokinesis in response to the treatment. Although the mRNA expression profile alone does not allow us to directly determine the role of any specific protein, especially those involved in phosphorylation/dephosphorylation signaling cascades, the microarray-based prediction herein allows to investigate novel signaling pathways to elucidate the effect of rosemary polyphenols in colon cancer cells.

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Conflict of interest Authors declare no conflict of interest.

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