A preliminary investigation demonstrating the effect of quercetin on the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism in human CO115 colon-adenocarcinoma cells using DNA microarray

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The role of the natural dietary flavonoid chemical quercetin (an antioxidant) in the prevention and treatment of colon cancer is receiving a great deal of attention. However, little is known about the molecular mechanisms of action of this flavonoid. In the present study, whole genome DNA microarrays were used to evaluate the effect of quercetin on gene expression in the CO115 colon-adenocarcinoma cell line with the completely deleted chromosome 18 harbouring the SMAD4 tumour-suppressor gene related to colon carcinogenesis. The study demonstrated that quercetin, widely present in fruit and vegetables, inhibited the growth of CO115 cells at 100 μM concentration in both the G_1/S and the G_2/M phases by modulating cell-cycle and apoptosis-related genes. Differential changes in accumulation of transcripts analysed for cells treated with 100 μM quercetin for 24 and 48 h in three independent repeated experiments revealed 5060–7000 differentially expressed genes. This means that quercetin probably does have a broad modulatory effect on gene expression in colon cancer. Out of these differentially expressed genes, the expression of 35 and 23 unique set of genes involved in cell-cycle control, apoptosis and xenobiotic metabolism were significantly altered after 24 and 48 h quercetin treatment respectively. Our results represent a novel aspect of the biological profile of quercetin that induces cell-cycle arrest through modulation of cell-cycle-related and apoptosis genes. The present study demonstrates a new step in elucidating the underlying molecular mechanisms of the antitumour action of quercetin, which could become a chemopreventive or chemotherapeutic agent for colon cancer.

Introduction

Nutrigenomics, which may be defined as the application of genomic tools to study the integrated effects of plant-based nutrients and non-nutrients on gene regulation in the whole organism in health and disease, is evolving rapidly [1,1a]. A number of studies have reported that there is a strong correlation between high dietary intake of flavonoids especially quercetin (3,3′,4′,5,7-pentahydroxyflavone) in humans and a lower incidence of colon cancer in these populations [2]. Quercetin, a plant-based flavonoid, is one of the agents in Phase I clinical trials at the National Cancer Institute, Bethesda, MD, U.S.A., pending its evaluation for the prevention of colon and other cancers [3]. Enthusiasm for quercetin in cancer prevention stems from its showing a number of separate and independent mechanisms of antitumour action, especially through inhibiting, in Phase I clinical trials, the expression of a tyrosine kinase thought to be involved in oncogenesis via an ability to override normal regulatory growth [4]. Likewise, high concentrations of quercetin can inhibit the growth of malignant cells by arresting the cell cycle in the late-G_1-phase or cause apoptosis [5].

With a new miniaturized technology, DNA microarray, the expression of over 10 000 human genes can be measured in one analysis. This very powerful tool can be used to find new genes that are affected by different food components, especially those with anticancerous properties [6]. Preliminary animal and human studies have confirmed that quercetin does indeed have therapeutic activity in at least some cancer situations [4]. To our knowledge the expression profile of genes associated with response to quercetin in human colon-cancer cell lines using DNA microarray has not yet been reported. The present study was therefore carried out with the aim of using new and

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Abbreviations used: all of the abbreviations are defined in Table 1 and, for brevity, are not repeated here.

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genome-wide information on the gene expression profile induced by quercetin in colon-cancer cells to elucidate mechanisms involved in the cancer-preventive action of quercetin. In human colorectal cancer, allelic loss at 18q21.1 has been reported to occur in up to 60% of cases, which maps to *DCC* (deleted in colon carcinoma), *DPC4ySMAD4* (deleted in pancreatic cancer 4) and *SMAD2*. Subsequent studies have identified *SMAD4* as the major tumour-suppressor gene harboured in this region and involved in the pathogenesis and evolution of colorectal cancer [7,8]. The human CO115 colon-adenocarcinoma cell line used in the present study has completely deleted chromosome 18 harbouring the *SMAD4* tumour-suppressor gene [9].

In the present study, the CO115 colon-cancer cells, when exposed to two different concentrations (10 and 100 μM) of quercetin, demonstrated gene-expression changes measured at two exposure time points ranging from 24 to 48 h. This preliminary investigation demonstrates that quercetin can most probably inhibit the growth in the G_1/S and G_2/M phases of CO115 human colon-adenocarcinoma cells by modulating genes involved in cell-cycle arrest and apoptosis.

Materials and methods

Cell culture and quercetin treatment

CO115 human colon-cancer cells were cultured at 37 °C in a humid atmospheric chamber consisting of CO_2/air (1:19). Quercetin dehydrate (Aldrich) was dissolved in DMSO (Hybri-Max, Sigma). Cells were plated at a density of 2×10^4 cells/ml in 50-ml culture flasks and exposed either to 0.1% DMSO alone or to 10 μM (referred to as 'low concentration') or 100 μM quercetin (referred to as 'high concentration') for 24 or 48 h. The final DMSO concentration in the medium was 0.1%.

Analysis of cell death

To assay the effect of quercetin on cell growth, viability was determined for the CO115 cells using the Trypan Blue exclusion assay, with three independent experiments for each treatment. The final quercetin concentrations in the medium used were 10 and 100 μM. Tumour cells were seeded in 25 ml flasks with 2×10^4 cells/ml and divided into control and treatment groups. The control groups consisted of cells treated with 0.1% DMSO alone and treatment groups with 10 and 100 μM quercetin. After 24 and 48 h, cells were removed from each well, stained with Trypan Blue and counted under a microscope for viable and dead cells. The percentage of viable cells was estimated after counting 100 cells for each sample.

Extraction of total RNA and preparation of cRNA

Cells, both those after exposure in three independent experiments to 10 and 100 μM quercetin, and those not exposed to quercetin, were pelleted down and stored in RNALater® (P/N 7020, Ambion). Total RNA was isolated from cells using RNeasy Mini Kit (P/N 74104, Qiagen) according to the manufacturer's protocol. The quality of the isolated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies) and a Bioanalyzer 2100 (Agilent). Only those samples with a A_{260}/A_{280} ratio between 1.8 and 2.1 and a 28 S/18 S ratio within 1.5–2 were further processed. Total RNA samples (5 μg) were reverse-transcribed into double-stranded cDNA with One-Cycle cDNA Synthesis Kit (P/N 900431, Affymetrix Inc.). The double-stranded cDNA was purified using a Sample Cleanup Module (P/N 900371, Affymetrix). The purified double-stranded cDNA was *in-vitro*-transcribed in the presence of biotin-labelled nucleotides using an IVT Labeling Kit (P/N 900449, Affymetrix). The biotinylated cRNA was purified using a Sample Cleanup Module (P/N 900371, Affymetrix) and its quality and quantity was determined using a NanoDrop ND 1000 and a Bioanalyzer 2100.

Array hybridization

Biotin-labelled cRNA samples (15 μg) were fragmented randomly to 35–200 bp at 94 °C in Fragmentation Buffer (P/N 900371, Affymetrix) and were mixed in 300 μl of hybridization buffer containing a hybridization control cRNA and Oligo B2 control (P/N 900454, Affymetrix Inc.,), 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated BSA in Mes buffer, pH 6.7, before hybridization to GeneChip® Human Genome U133 Plus 2.0 arrays for 16 h at 45 °C. Arrays were then washed using the Affymetrix Fluidics Station 450 EukGE-WS2v5_450 protocol. An Affymetrix GeneChip Scanner 3000 was used to measure the fluorescent intensity emitted by the labelled target.

Statistical analysis

Raw data processing was performed using the Affymetrix GCOS 1.2 software. After hybridization and scanning, probe cell intensities were calculated and summarized for the respective probe sets by means of the MAS 5-algorithm [10]. To compare the expression values of the genes from chip to chip, global scaling was performed, which resulted in the normalization of the trimmed mean of each chip to a target intensity (TGT value) of 500 as detailed in the statistical algorithms description document 2002 (Affymetrix). Quality-control measures were considered before performing the statistical analysis. These included adequate scaling factors (between 1 and 3 for all samples) and appropriate numbers of present calls calculated by application of a signed-rank call algorithm [11]. The efficiency

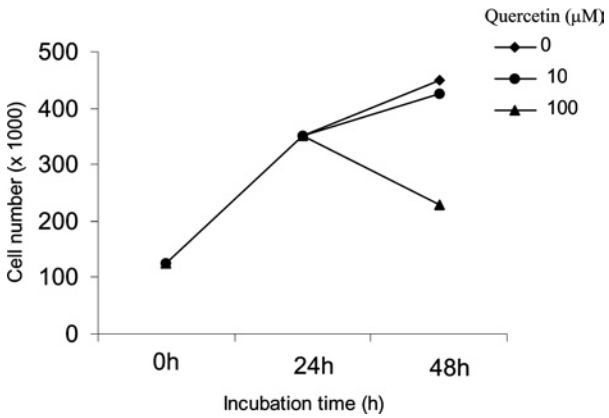

Figure 1 Effect of two different concentrations of quercetin on cell growth (cell viability) of human CO115 colon-adenocarcinoma cells

The cells were incubated with the flavonoid for 48 h. The value are means ± S.E.M. for three different experiments.

of the labelling reaction and the hybridization performance was controlled with the following parameters: present calls and optimal 3′/5′ hybridization ratios (around 1) for the housekeeping genes [*GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *ACO7*], for the poly(A) spike in controls and the prokaryotic control (*BIOB*, *BIOC*, *CREX*, *BIODN*). The data were further normalized and processed with the gcRMA algorithm (robust multichip average) using remote analysis computation for gene expression data ('RACE', University of Lausanne, Lausanne, Switzerland) as well as Gene Spring Software (Agilent Technologies).

Results

Effect of quercetin on cell growth

Figure 1 shows the effect of 10 (low concentration) and 100 μM (high concentration) quercetin concentrations on growth (cell viability) of CO115 cells measured by the Trypan Blue exclusion assay. The cells were incubated with the flavonoid for 48 h. It was noteworthy that exposure of CO115 cells to the low concentration of quercetin for 48 h and to the high concentration of the chemical for 24 h had the least effect on cell viability. It was observed that cells so treated behaved more or less the same as untreated or control cells (cells treated with 0.1 % DMSO). However, treatment of CO115 cells with the high concentration of quercetin for 48 h significantly suppressed the cell growth. Interestingly, a more-than-30 % decrease in cellular viability of CO115 cells was observed after 48 h incubation in 100 μM quercetin (Figure 1). These results thus suggest that CO115 cells seemed to be most susceptible to this concentration of quercetin after 48 h exposure. On the other hand, no significant differences in cell number were found between untreated and vehicle controls (results not shown). The results thus further suggest that DMSO alone at a concentration of 0.1 % did not modify cell growth.

Quercetin modulates expression of cell-cycle regulatory genes

To elucidate the mechanism(s) responsible for the reduction in viable cell number with time by quercetin treatment in CO115 colon-cancer cell lines, differential expression of cell-cycle-related genes were examined after 24 as well as after 48 h. In the present study, the low (10 μM) concentration of quercetin was unable to cause any significant change in the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism (results not shown). However, 100 μM quercetin significantly altered the expression of genes involved in the abovementioned functions (Tables 1 and 2). At this concentration of chemical, a significant increase was observed in the expression of early-response genes (24 h quercetin exposure) related to cell-cycle arrest in G_1/S and G_2/M phases that included *CDKN2B* (p15), *SESN2*, *CDKN1C* (p57) and *CDKN1A* [CDK (cyclin-dependent-kinase) inhibitor, p21], and the same concentration caused down-regulation of *PLK1*, *CDK4*, *BUB1B* and *CDC20* cell-cycle-promoting genes (Table 1). Early- or late-response genes were defined as genes that were differentially expressed after exposure to 100 μM concentration of quercetin for 24 and 48 h respectively. Among the early-response genes, interestingly, quercetin was able to up-regulate the expression of the *p21* gene without the induction of the p53 gene and, at the same concentration, expression of *CDKN2B* (*p15*) was also strongly up-regulated (2.1-fold) in SMAD4-devoid CO115 cells after 24 h exposure. Among the late-response cell-cycle-inhibiting genes, *TNFSF15* and *RGS5* were strongly up-regulated (> 2-fold) and cell-cycle-promoting *E2F1*, *RASGR2*, *CDK2*, *TCF19*, *FGD2*, *CDC25A* and *CAMK2D* were strongly down-regulated (> 2-fold) on quercetin treatment (Table 2). Surprisingly, VEGF (vascular epithelial growth factor), an angiogenesis factor, was strongly up-regulated (2.5-fold) after 24 h exposure to high concentrations of quercetin. Quercetin was able to influence the expression of genes involved in both the G_1/S and G_2/M phases of the cell cycle, indicating that they inhibit the growth of most of the malignant cells by arresting them in both of these phases of the cell cycle.

Quercetin modulates the expression of apoptosis-related genes

Since CO115 cells exhibited a high sensitivity to the effect of quercetin by differential expression of cell-cycle-related genes, we investigated possible mechanisms by which this

Table 1 Differential expression of some important apoptotic and cell-cycle-related genes in CO115 cells after 24 h exposure to 100 μM quercetin

Probe set ID	Gene title	Gene symbol	Fold change ($n = 3$)	Involved in
210609_at	Tumour protein p53 inducible protein 3	*TP53I3*	1.51	Induction of apoptosis
225912_at	Tumour protein p53 inducible nuclear protein 1	*TP53INP1*	2.43	Induction of apoptosis
210334_at	Baculoviral IAP repeat containing 5 (survivin)	*BIRC5*	−1.51	Anti-apoptosis
207530_at	Cyclin-dependent kinase inhibitor 2B (p15)	*CDKN2B*	2.1	Cell-cycle arrest
223196_at	Sestrin 2	*SESN2*	1.55	Cell-cycle arrest
202240_at	Polo-like kinase1	*PLK1*	−1.56	Regulation of cell cycle
203755_at	budding uninhibited by benzimidizole 1B	*BUB1B*	−1.50	Cell-cycle spindle organization
204151_at	Aldo–keto reductase family 1, member C1	*AKR1C1*	3.48	Xenobiotic metabolism
201468_at	NAD(P)H dehydrogenase quinone 1	*NQO1*	1.51	Xenobiotic metabolism
236140_at	Glutamate:cysteine ligase modifier subunit	*GCLM*	1.57	Glutathione biosynthesis
200779_at	Activating transcription factor 4	*ATF4*	1.61	Regulation of transcription
202017_at	Epoxide hydrolase 1 microsomal (xenobiotic)	*EPHX1*	1.63	Xenobiotic metabolism
212173_at	Adenylate kinase 2	*AK2*	−1.58	Nucleic acid metabolism
222305_at	Hexokinase 2 (glycolysis)	*HK2*	−1.52	Regulation of cell-cycle glycolysis
203691_at	Protease inhibitor	*PI3*	3.21	Serine-type-endopeptidase inhibitor activity
213756_at	Heat-shock transcription factor	*HSF1*	−1.55	Regulation of transcription
205409_at	Fos-like antigen 2	*FOSL2*	−1.90	Regulation of transcription
239288_at	TRAF2- and NCK-interacting kinase	*TNIK*	−151	Regulation of translation
202246_at	Cyclin-dependent kinase 4	*CDK4*	−1.50	Regulation of cell cyde
202870_at	CDC20 cell division 20 homologue	*CDC20*	−1.56	Regulation of cell cycle
221478_at	Bcl2/adenovirus E1B 19 kDa interacting protein 3-like	*BNIP3L*	2.73	Induction of apoptosis
221480_at	Heterogenous nuclear ribonucleoprotein D	*HNRPD*	−1.54	Telomere maintenance
202086_at	Myxovirus (influenza virus) resistance 1 (interferon-inducible protein p78)	*MX1*	1.52	Induction of apoptosis
210513_at	Vascular endothelial growth factor	*VEGF*	2.5	Regulation of cell cycle
204025_at	Programmed cell death 2	*PDCD2*	−1.2	Induction of apoptosis
228253_at	Lysyl oxidase-like 3	*LOXL3*	1.7	Oxidoreductase activity
204731_at	Transforming growth factor-β receptor III	*TGFBR3*	1.51	Signal transduction
210654_at	Tumour-necrosis-factor receptor superfamily member 10 D	*TNFRSF10D*	2.3	Induction of apoptosis
213348_at	Cyclin-dependent kinase inhibitor 1C (p57)	*CDKN1C*	1.5	Cell-cycle arrest
154436_at	Regenerating islet-derived family member 4	*REG4*	−2.12	Sugar binding
202284_at	Cyclin-dependent kinase inhibitor 1A (p21)	*CDKN1A*	1.53	Regulation of cell cycle

flavonoid might interfere with the apoptosis process in this cell line. In the present study, in an attempt to explore the effect of quercetin on apoptosis-regulatory proteins, we examined the expression of both apoptotic and anti-apoptotic genes. High numbers of genes involved in apoptosis were found to be differentially expressed in CO115 cells in response to the highest dose of quercetin (Tables 1 and 2). Six early-response genes that were differentially expressed in CO115 cells on treatment with high concentrations of quercetin included *TP53I3*, *TP53INP1*, *BIRC5*, *BNIP3L*, *MX1*, *TNFRSF10D* and *PDCD2*. The most striking effect among the early-response genes was observed in the pro-apoptotic *BNIP3L*, *TP53INP1* and *TNFRSF10D* genes, which were strongly up-regulated, namely 2.73-, 2.43- and 2.3-fold respectively. Likewise, the late-response pro-apoptotic genes, including *BAX*, *CASP8* (caspase 8), *BCL2L11*, *TNFRSF6*, *APAF1* and *PDCD4* (programmed cell death 4), were up-regulated, and some anti-apoptotic genes such as *HELLS* (helicase, lymphoid-specific) were down-regulated on quercetin treatment. The *PDCD4* and *BAX* genes were strongly up-regulated 1.9- and 1.7-fold respectively after 48 h exposure to the high concentration of quercetin. Interestingly, we could not observe in CO115 cells any significant change in the expression of the pro-apoptotic *p53* gene, though there was a remarkable change in the expression profile of its primary and secondary targets on quercetin treatment.

Response of genes involved in Phase-I and Phase-II metabolism to quercetin treatment

Since quercetin is an antioxidant, in the present study the expression profile of genes involved in xenobiotic metabolism was also simultaneously examined in CO115 cells exposed to quercetin. Among the early-response xenobiotic-metabolism-related genes, *ACK1C1* (aldo–keto reductase family 1 member C1), *NQO1* [NAD(P)H dehydrogenase quinone 1], *EPHX1* (epoxide hydrolase 1), *LOXL3*, *TXNR1* (thioredoxin reductase) and that coding for the light regulatory subunit of GCLM (γ-glutamylcysteine synthetase) were strongly up-regulated on high-quercetin treatment. Quercetin was able to produced a striking induction of *AKR1C1*, which became 3.48-fold up-regulated after quercetin exposure. Surprisingly, two late-response xenobiotic-metabolism genes, *CYP4F11* and *CYP3A5*, which encode proteins for detoxification, were highly expressed (4.72- and

Table 2 Differential expression of some important apoptotic and cell cycle related genes in CO115 cells after 48 h exposure to 100 μM of quercetin

Probe set ID	Gene title	Gene symbol	Fold change (treated/control) ($n = 3$)	Involved in
2028_S_at	E2F transcription factor 1	*E2F1*	−2.1	Regulation of cell cycle
208478_at	Bcl2 associated X protein	*BAX*	1.58	Induction of apoptosis
209917_at	TP53 activated protein 1	*TP53AP1*	1.51	Response to stress, apoptosis
213373_at	Caspase 8	*CASP 8*	1.53	Induction of apoptosis
223556_at	Helicase lymphoid specific HELLS	*HELLS*	−1.61	Helicase activity (apoptosis)
222343_at	Bcl2-like 11	*BCL2L11*	1.56	Induction of apoptosis
215719_at	Fas (TNF receptor superfamily member 6)	*TNFRSF6*	1.57	Apoptosis
201841_at	Heat-shock protein β1 (HSP27)	*HSPB1*	−1.52	Stress resistance and actin organization
214368_at	Ras guanyl-releasing protein 2	*RASGR2*	−1.50	Regulation of cell growth
204252_at	Cyclin-dependent kinase 2	*CDK2*	−1.59	Regulation of cell division
222003_at	Dedicator of cytokinesis 6	*DOCK6*	1.61	GTP binding
202730_at	Programmed cell death 4	*PDCD4*	1.93	Induction of apoptosis
223274_at	Transcription factor 19(Sc1)	*TCF19*	−1.57	Regulation of cell cycle
209071_at	Regulation of G-protein signalling 5	*RGS5*	1.53	Regulation of signal transduction
221085_at	Tumour necrosis factor (ligand) superfamily member 15	*TNFSF15*	2.20	Regulation of cell cycle
1553906_at	FYVE, Rho GEF and PH domain containing 2	*FGD2*	−1.23	Regulation of cell shape
230134_at	Member-associated DNA-binding protein	*MNAB*	1.55	Protein ubiquitination
226613_at	TBC1 domain family member 10A	*TBC1D10*	2.04	Protein binding
213317_at	Chloride intracellular channel	*CLIC5*	1.68	Ion transport
230682_at	ATP-binding-cassette subfamily C member 3	*ABCC3*	1.65	Transport
232004_at	Heterogenous nuclear ribonucleoprotein R	*HNRPR*	−1.58	mRNA processing
204971_at	Cystatin A (stefin A)	*CSTA*	1.66	Cysteine-protease inhibitor activity
207935_at	Keratin 13	*KRT13*	1.77	Epidermis development
227803_at	Ectonucleotide pyrophosphate	*ENPP5*	1.55	Hydrolase activity
201802_at	Solute carrier family 29	*SLC29A1*	−1.49	Nucleoside transport
228964_at	PR domain containing 1 with ZNF domain	*PRDM1*	1.93	Regulation of transcription
205765_at	Cytochrome P450 family 3 subfamily A polypeptide 5	*Cyp3A5*	2.46	Electron transport, xenobiotic metabolism
206153_at	Cytochrome P450 family 4 subfamily F polypeptide 11	*Cyp4F11*	4.72	Electron export (mono-oxygenase activity)
217767_at	Complement component 3	*C3*	3.8	Complement activation
204695_at	Cell division cycle 25 A	*CDC25A*	−1.51	Mitosis
228555_at	Calmodulin-dependent protein kinase IIδ	*CAMK2D*	−2.1	Regulation of cell cycle
204859_at	Apoptotic protease-activating factor 1	*APAF1*	1.53	Regulation of apoptosis

2.46-fold respectively) in response to 100 μM quercetin (Table 2).

Modulation of some other important genes by quercetin treatment

In addition, some other important early- and late-response genes, such as *ATF4*, *C3*, *AK2*, *PI3*, *HSF1*, *HNRPD*, *TGFRBR3*, *RASGR2*, *FOSL2* and *REG4*, were also differentially expressed on quercetin treatment (Tables 1 and 2). The expression of an important heat-shock protein, HSPB1, was also observed to become down-regulated on quercetin treatment.

Discussion

The present study suggests that quercetin may act as a potent growth-inhibitory compound in the CO115 colon-cancer cell line, and the effectiveness of quercetin in inhibiting cell growth was observed much more at higher doses (100 μM) with prolonged exposure (48 h). Such results are in accordance with the observations that suggest that higher concentrations of quercetin are required for it to exhibit its inhibitory effects on the growth of various cancer cell lines as well as on different stages of tumour development in animal studies [5,12]. The findings also support the possibility of chemopreventive or chemotherapeutic potential for this flavonoid in colon cancer [13].

To gain an insight into the potential mechanisms by which quercetin inhibits cancer cell growth and as such acts as a putative cancer-preventive agent, we examined the gene-expression pattern in CO115 colon-cancer cell lines after treatment with different concentrations of quercetin in parallel with untreated cells at different time periods. CO115 are devoid of chromosome 18, the chromosome that harbours very important genes, including the *SMAD4* tumour-suppressor gene which affects cell-cycle control {cells do not respond to TGF-β (transforming growth factor-β) signalling and eventually escape from TGF-β-mediated growth control and apoptosis, causing transformation [14,15]}. Comparatively, 10 μM quercetin treatment was unable to cause a significant change in the expression of genes related to cell-cycle arrest, apoptosis and xenobiotic metabolism, and thus, in the present study, we considered only differential expression of the above-mentioned genes in response to the highest dose (100 μM) of quercetin. Some recent reports have suggested that

quercetin becomes completely degraded after 24 h in the culture medium if it is not protected by ascorbate. Therefore, in the present study, the low concentration (10 μM) may have resulted in minimal response.

In the present study, out of a total of 5060–7000 differentially up-regulated or down-regulated early- and late-response genes in CO115 cells, on exposure to a high concentration of quercetin, only those genes that were involved within a pathway or at pathways known to be important in colon carcinogenesis, life-cycle control apoptosis and xenobiotic metabolism were selected for further analysis. Interestingly, after exposure of cells to a higher dose of quercetin, more than 35 early- and 23 late-response genes related to cell-cycle arrest, apoptosis, xenobiotic metabolism and signal transduction were differentially expressed ($>$1.5-fold, $P < 0.05$), as reported previously for other plant chemicals [16].

Quercetin has been reported to inhibit the growth of various cell lines derived from human cancers by blocking their cell division at different points (G_1/S and G_2/M phase), depending on the tumour origin and the cell type [3,12,17,18]. Therefore, in the present study, a search for genes related to different phases of cell cycle and demonstrating differential modes of expression on quercetin treatment, were analysed. Among the early-response genes involved in G_1/S phase of the cell cycle, *p15*, *p21*, *p57* and *SESN2* were up-regulated and, at the same time, cell-cycle-promoting genes, such as *CDK4*, were down-regulated on quercetin treatment. Induction of p21 has been reported as being responsible for growth inhibition in colon-cancer cells, including Tys cells [19]. It is noteworthy that quercetin was able to cause induction of p21 independently of wild-type p53-tumour-suppressor gene product expression in the CO115 cell line. Similarly quercetin treatment independently induced the kinase inhibitor p15 in SMAD4-devoid CO115 cells. SMAD4 forms the core component of the TGF-β signal-transduction pathway regulating the expression of cyclin-dependent kinase inhibitors, including p15, and its loss allows the cell to escape from growth control and apoptosis [20]. Therefore the current observations suggest that quercetin may play a very potent role as an antitumour agent causing cell-cycle arrest in colon-cancer cells without the involvement of p53 or the presence of SMAD4-tumour-suppressor genes.

The late-response *TNFSF15* gene involved in the G_1/S phase of the cell cycle, which is reported to suppress proliferation of murine colon cancer, was strongly up-regulated ($>$2 fold) in the present study on exposure to quercetin. At the same time, cell-cycle-promoting *E2F1*, *CDK2*, *CAMK2D* and *CDC25A* genes were strongly down-regulated on quercetin treatment. Our study clearly indicates that quercetin possesses a potent kinase-inhibitory action, simply by up-regulating most of the tyrosine and protein kinase inhibitors, especially *CDKN2B* and *CDKN2A1*, as well as down-regulating the *CDK4* and *CDK2* genes involved in the G_1/S phase of the cell cycle. Thus it is suggested that these protein kinase modulators, present in food items of plant origin, target a large number of both tyrosine and serine/threonine protein kinases involved in the signal-transduction pathways of cellular growth and differentiation and thus cause the cell-cycle arrest [4].

Regarding the genes related to the G_2/M phase of the cell cycle, quercetin was able to modulate cell-cycle-promoting *PLK1*, *CDC20*, *TCF19*, *FGD2* and *BUB1B* genes by their down-regulation, thus suggesting that it can also cause cell-cycle arrest in this phase of the cell cycle. The *PLK1* gene product that induces mitosis has been reported to be expressed at higher levels in colorectal cancer than in normal colon tissue. Down-regulation of this gene on quercetin treatment, as seen in our present study, may indicate the same mechanism of action as reported previously for curcumin treatment, leading to G_2/M-phase arrest of cancer cells by down-regulation of this gene [16,21]. Likewise, *BUB1B*, *FGD2* and *TCF19* genes, which play a central role in mitosis, either in the spindle checkpoint involved in correct chromosome segregation during cell division, or in regulation of cell shape, was also down-regulated on exposure of CO115 cells to quercetin. These results indicate that quercetin acts as a potent inhibitor of these cell-cycle-related novel anticancer drug targets. Furthermore, the study provides evidence that quercetin is involved in cell-cycle arrest in both the G_1/S and G_2/M phases through modulation of related genes.

Interestingly, in contradiction of earlier reports in our studies, VEGF, an angiogenesis factor involved in cell-cycle progression, was highly up-regulated in response to quercetin [22]. Such observations suggest that up-regulation of VEGF might be a rescue reaction by colon-cancer cells under quercetin stress to gain VEGF from outside by angiogenesis, but this requires further investigation [23].

In order to determine, in the present study, whether quercetin was able to inhibit the growth of a human colon-adenocarcinoma cell line through mechanisms involving the induction of apoptosis, expression of genes involved in different apoptotic molecular pathways were analysed. Apoptosis is an orderly mechanism of cellular self-destruction that can lead to the selective deletion of damaged cells from tissues. Various natural chemopreventive agents induce apoptosis via several pathways, including DNA damage through p53-independent and p53-dependent pathways [24]. Interestingly, in our model, quercetin treatment induced a maximum number of pro-apoptotic genes and also caused down-regulation of important anti-apoptotic genes ($>$1.5-fold). Previous studies have demonstrated that most anticancer agents, such as 5-fluorouracil, induce apoptosis through a p53-dependent pathway and are

ineffective in p53 mutant tumours [25]. However, in contradiction to such reports, the present study showed that quercetin was unable to directly induce wild-type p53 in CO115 colon-cancer cells, but, at the same time, modulated more than a few known primary and secondary p53 targets, including pro-apoptotic PDCD4, p21, BAX, APAF1 etc., on quercetin treatment [26]. Thus it can be suggested that, most probably, induction of apoptosis by quercetin in our model is independent of the presence or induction of the p53 gene product and the process may be initiated via modulation of other gene transcripts that contribute to p53-independent apoptosis [27]. It is noteworthy that our study revealed, for the first time, the differential expression of more than 24 transcripts, most of them including the genes that are either primary or secondary targets of p53, and this suggests that alternative pathways of apoptosis are involved in cancer cells on quercetin treatment. Some of the important early-response pro-apoptotic genes, namely *BNIP3L*, *TP53INP1* and *TNFRSF10D*, were strongly up-regulated (2.73-, 2.43- and 2.3-fold respectively) and, at the same time, some late-response pro-apoptotic transcripts, namely *BAX*, *TP53IA*, *CASP8*, *BCL2L11*, *TNFRSF6*, *CDKN1A* and *PDCD4*, are also highly up-regulated on exposure to high doses of quercetin. Among these up-regulated pro-apoptotic genes that are involved in alternative pathways of apoptosis, activation of APAF1, a novel protein induced by quercetin, can directly participate in cytochrome *c*-dependent activation of CASP3, which triggers a cascade of events in apoptosis [28]. Likewise, BNIPL, which acts as a negative regulator of survival gene product activity, and TNFRSF10D, which causes a form of programmed cell death, trigger the activity of proteolytic caspases whose actions dismantle the cell and thus result in the cell death. Further, our study also demonstrates the strong expression of the *TP53INP1* gene ($>$2.4-fold) on quercetin treatment, and the role of this gene has recently been shown to be to induce apoptosis through an alternative p73-mediated pathway in p53-deficient or mutated cells [29]. Thus our results clearly indicate that quercetin is, firstly, involved in the p53-independent regulation of cell-cycle progression in CO115 colon cancer cells and, at the same time, compels them to adopt an alternative pathway of induction of apoptosis in association with the p73 gene without any visible up-regulation of the p53 gene.

Previous reports suggest that, unlike normal cells, many cancer cells, including colon-cancer cells, are relatively resistant to CD95-mediated cell death [12]. However, one of the ways to overcome such resistance in such cases and enhance apoptosis is either by directly activating CASP8, as observed in the present study, or by activating alternative pro-apoptotic pathways parallel with, or independent of, CD95.

Overexpression of HSPs can also make tumour cells very resistant to apoptosis [30]. Therefore one of the ways to induce apoptosis in such tumours is achieved by decreasing HSPs proteins levels within them. Our study demonstrated that quercetin is able to decrease the expression of a small HSP (hsp27) that has been correlated with growth and drug resistance in some human cancer cell lines [31]. Thus, in the present study, activation of *CASP8*, *Apaf1*, *BAX* (etc.) genes provides additional evidence of more than one p53-independent apoptotic pathway involved in colon cancer cells on quercetin treatment. In addition, our results provide evidence of adapting alternative mechanisms of pro-apoptotic activity by colon cancer cells in response to quercetin treatment through down-regulation of HSPs [32].

The observation that quercetin-dependent cell-cycle arrest in colon-cancer cells is not apparently related to p53 expression is extremely important from a therapeutic point of view, since most anticancer agents, such as 5-fluorouracil, induce apoptosis directly through wild-type p53 and are ineffective on p53-devoid or p53-mutant tumours [25].

Epidemiological data converge and affirm that regular use of naturally occurring antioxidants present in fruits and vegetables reduces the risk of colon cancer in the population [2]. In the present study it was found that quercetin can regulate the expression of some important antioxidant-regulated genes in human CO115 colon-cancer cell lines. Most of these modulated genes, namely *AKRIC1* (dihydrodiol dehydrogenase), *NQO1*, *EPHX1* and *TXNRD12*, involved in Phase II metabolism, became strikingly up-regulated on quercetin treatment. Among these Phase II genes, the *AKR1C1* gene, which is underexpressed in 50% of gastric cancers, was found to be strikingly up-regulated on quercetin treatment, as reported previously for some phenolic antioxidants, including isothiocyanates [16,33,34]. Likewise it was surprising that, among the late-response xenobiotic-metabolism genes, *CYP4F11* and *CYP3A5*, which code for detoxification proteins, were highly expressed ($>$4.4-fold) in CO115 cells on quercetin treatment. These findings suggest that cells exposed to quercetin obtained through diet have higher levels of Phase I as well as Phase II detoxification enzymes and thereby indicate an increased level of protection of the cells against oxidative stress. Such cells are also better prepared for subsequent toxic insults.

In summary, the natural polyphenolic quercetin may act as a strong growth-inhibitory compound in human colon cancer through modulation of primary or secondary targets of p53 transcripts, as well as other genes involved in alternative apoptotic pathways, and these findings support the possibility of using this flavonoid in the chemoprevention and chemotherapy of colon cancer. The present study gives an insight into new molecular targets that may also serve to access the efficacy of other potential

chemopreventive agents besides quercetin for prevention of colon cancer by diet-derived compounds.

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