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ORIGINAL CONTRIBUTION

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Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro

Summary *Background* Many different mechanisms are involved in nutrient-related prevention of colon cancer. In this study, a comprehensive assessment of the spectrum of possible biological actions of the bioactive compound quercetin is made using multiple gene expression analysis. Quercetin is a flavonoid that can inhibit proliferation of tumor cells and reduce the number of aberrant crypt foci, although increase of number of colon tumors was also reported. *Aim of the study* In order to elucidate possible mechanisms involved in its mode of action the effect of quercetin on expression of 4000 human genes in Caco-2 cells was studied and related to functional effects. *Methods* Caco-2 cells were exposed to 5 or 50 µM quercetin for 48 hours, differential expression of 4000 human genes was studied using microarrays and related to functional effects. Differentially expressed genes were categorized in seven functional groups: cell cycle and differentiation, apoptosis, tumor suppressor genes and oncogenes, cell adhesion and cell-cell interaction, transcription, signal transduction and energy metabolism. Also, cell proliferation and cell cycle distribution were measured. *Results* Quercetin (5 µM) downregulated expression of cell cycle genes (for example CDC6, CDK4 and cyclin D1), downregulated cell proliferation and induced cell cycle arrest in Caco-2 cells. After exposure to 50 µM quercetin cell proliferation decreased to 51.3 % of control, and further decrease of the percentage of cells in the G1 phase coincided with an increase of the percentage of cells in the sub-G1 phase. Quercetin upregulated expression of several tumor suppressor genes. In addition, genes involved in signal transduction pathways like beta catenin/TCF signalling and MAPK signal transduction were influenced by quercetin. *Conclusions* This study shows that large-scale gene expression analysis in combination with functional assays yields a considerable amount of information on (anti-)carcinogenic potential of food components like quercetin.

Key words quercetin – Caco-2 – gene expression – cell proliferation – (anti-)carcinogenic mechanisms

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Introduction

Diet plays an important role both in the development and prevention of colon cancer. Many food components have been reported to protect against colon cancer through a variety of (proposed) mechanisms. However, a body of seemingly conflicting evidence on these food bioactives has been published, suggesting that elucidation of colon cancer-preventive mechanisms is not always straightforward. Depending on dose levels, uptake rates and also the model used different results can be obtained. New techniques, like multiple gene expression analysis employing microarrays, allow for a more com-

prehensive study of the effects and mechanisms of food components. By using cDNA microarrays the expression of thousands of genes can be studied in one experiment. The power of these techniques lies in the fact that a large set of real experimental data is acquired, which can result in leads to pathways and mechanisms, as opposed to the extrapolation of assessments of a few genes. Microarrays have been used for example to study gene expression profiles of human colon cancer biopsies from different disease stages [1], and of colon cancer cells after exposure to NSAIDs and butyrate [2, 3]. Such experiments will lead to a better understanding of the process of colon carcinogenesis and of prevention of colon cancer development by food components.

Quercetin is a well-known flavonoid present in plants, consumed in relatively high amounts [4] and with multiple health effects. Quercetin is a potent antioxidant and has presumed anti-inflammatory effects. Quercetin has shown a range of effects in *in vitro* and *in vivo* studies. In rats, quercetin has been found to reduce the number of aberrant crypt foci (ACF) after chemical induction of tumor formation [5, 6], but quercetin has also been reported to dose-dependently increase the number of colon tumors after azoxymethane (AOM) injection [7]. In mice, quercetin reduced the number of focal areas of dysplasia (FAD) induced by AOM [8, 9] and also the number of colon tumors and tumor multiplicity [8]. However, when quercetin was administered without AOM, it caused an increase in FADs in mice [9]. In mice with a germline APC mutation, quercetin had no effect on intestinal tumor formation [10]. Thus, quercetin displayed both anti-carcinogenic and carcinogenic properties, depending on model and concentration used.

Quercetin was shown to inhibit growth of tumor cells in vitro [11, 12], can be cytotoxic for actively proliferating cells [13] and can cause undifferentiated cancer cell lines to differentiate [14]. Quercetin is unstable in cell culture media and disappears quickly [15]. Kuo et al. found that quercetin was unstable, but still exerted an antiproliferative effect in Caco-2 and HT29 cells, which in part may be caused by its metabolites [12]. Murota et al. showed that in Caco-2 cells quercetin is efficiently absorbed and metabolized [16].

In bacteria quercetin has mutagenic potential, as was shown by Bjeldanes [17]. In Caco-2 cells however, quercetin did not cause DNA strand breaks [18]. Several *in vitro* studies showed that quercetin can protect against hydrogen peroxide induced DNA strand breaks in Caco-2 cells [19, 20].

In fruits and vegetables quercetin is present as a glycoside (bound to sugar). To some extent these glycosides can be absorbed in the small intestine. The glycosides that reach the colon are hydrolyzed by the colonic microflora [4]. The large intestine can thus be exposed to relatively high concentrations of quercetin. Recently, van der Woude et al. stated that concentration of free quercetin can reach up to 100 μM in the intestinal lumen after ingestion of a quercetin supplement (250–500 mg) [21].

Thus, quercetin has a broad range of effects in colon cancer cells, but the exact mechanism is unknown. Studying the effect of quercetin on expression of thousands of genes is likely to give more insight into the bioactive mechanisms of this flavonoid. Therefore, in this study the effect of quercetin on expression of 4000 genes in human colon cancer cells is studied, simultaneously analyzing cell cycle, apoptosis, cell adhesion, transcription, signal transduction and tumor suppressor gene and oncogene responses to quercetin. In addition, to demonstrate the physiological relevance of the presented gene expression data cell proliferation and cell cycle distribution were measured, as cancer-related physiological end-points.

Materials and methods

Cell culture

Caco-2 cells (passage 38) (ATCC, Rockville, USA) were grown in DMEM with 25 mM HEPES, 10 % FCS, 1 % non-essential amino acids, 2 mM L-glutamine and 50 μg/ml gentamicine. After seeding, cells reached 100 % confluency in 7 days. Cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. Quercetin (Sigma, St. Louis, MO, USA) was dissolved in ethanol. Caco-2 cells were exposed to 5 or 50 μM quercetin, or only to ethanol (solvent). The final concentration of ethanol in the culture medium was 1 %. After 24 hours, fresh medium and quercetin were added to the cells, and 24 hours later cells were trypsinized and flash frozen in liquid nitrogen. All incubations were performed in quadruplicate. After 48 hours exposure, cells were counted and cytotoxicity was assessed using Trypan Blue exclusion.

Cell proliferation

After exposure of confluent Caco-2 cells to quercetin, cell proliferation was measured using an ELISA-BrdU-kit (Roche Diagnostics GmbH) to quantify bromodeoxy uridine (BrdU) incorporation. Absorbance at 370 nm was corrected for absorbance at 492 nm. In addition, this value was corrected for background absorbance. Absorbance of cells exposed to ethanol (solvent) was set to 100 %.

Cell cycle analysis using flow cytometry

After exposure of confluent Caco-2 cells to quercetin, cells were trypsinized and washed with PBS. Ice-cold ethanol (75 %) was added to the cells while vortexing, and cells were incubated on ice for 1 hour. Cells were washed with PBS and finally PBS with propidium iodide (Sigma; 50 µg/ml) and Rnase A (Qiagen, Hilden, Germany; 0.1 mg/ml) was added. After incubation for 30 min in the dark, cells were analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter). P-values for difference between cell cycle distribution in cells exposed to ethanol and in cells exposed to quercetin were calculated using a t-test.

RNA isolation

Total RNA was isolated from the cell pellets using Trizol (Life Technologies S. A., Merelbeke, Belgium) according to the manufacturer's protocol. RNA was checked for purity and stability by 1 % agarose gel electrophoresis and UV spectrometry. Absorption at 260 and 280 nm was measured spectrophotometrically and RNA quantity and $A_{260/280}$ ratio were calculated. Only RNA samples with $A_{260/280}$ ratio > 1.6 were used in further experiments.

cDNA microarray preparation

A set of about 4100 sequence-verified human cDNA clones from the I. M. A. G.E. consortium was purchased (Research Genetics, U. S. A.) as PCR products. The amplified cDNA was re-amplified by PCR with forward (5' -CTGCAAGGCGATTAAGTTGGGTAAC-3') and reverse (5'-GTGAGCGGATAACAATTTCACACAGGAAACAGC-3') primers. The primers contained a 5'- C6-aminolinker (Isogen Bioscience, Maarsen, The Netherlands) to facilitate crosslinking to the aldehyde coated glass microscope slides. PCR products were checked by electrophoresis on a 1 % agarose gel. PCR products were purified by isopropanol precipitation and washing in 70 % ethanol, and were dissolved in 3 x SSC. The clones were spotted in duplicate on CSS-100 silylated aldehyde glass slides (TeleChem, Sunnyvale, CA, USA) in a controlled atmosphere. To reduce free aldehyde residues, slides were blocked with borohydride after spotting and drying. Slides were stored at room temperature in the dark and dust-free until further use.

Quantitative real-time polymerase chain reaction

2 µg of total RNA was reverse transcribed into cDNA using 250 ng random hexamer primers and 200 units M-MLV reverse transcriptase (Invitrogen Life Technologies, Breda, the Netherlands) in a final volume of 20 µl. The same batch of cDNA was used for all real-time PCR experiments. Real-time PCR was performed using an iCycler PCR machine (Biorad, Veenendaal, the Netherlands) and the QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). For all types of amplicon primer concentration was 400 nM and reactions were performed in a final volume of 25 µl containing primers, cDNA and 1x QuantiTect SYBR Green Master Mix (Qiagen, Hilden, Germany). GAPDH and beta-actin expression was measured to correct for differences in efficiency during reverse transcription. The primer pairs used to amplify beta-actin, GAPDH, RALA, CDK4 and CYP1A1 were as follows: beta-actin upstream: 5'-CAC CCC GTG CTG CTG AC-3', downstream: 5'-CCA GAG GCG TAC AGG GAT AG-3'; GAPDH upstream: 5'-TGC ACC ACC AAC TGC TTA GC-3', downstream: 5'-GGC ATG GAC TGT GGT CAT GAG-3'; RALA upstream: 5'-GGT CAG AAT TCT TTG GCT TTA CAC A-3', downstream: 5'-CTC CCC ATC TAG CAC TAC CTT CTT C-3'; CDK4 upstream: 5'-GAG GCC TTC CCA TCA GCA CAG TTC-3', downstream: 5'-TCA AAA GCC TCC AGT CGC CTC AGT A-3'; CYP1A1 upstream: 5'- CCA TGT CGG CCA CGG AGT TTC-3', downstream: 5'- CAT GGC CCT GGT GGA TTC TTC A-3'.

Before real-time PCR analyses, the correct size of the obtained PCR products was checked by gel electrophoresis. At the end of all real-time PCR runs, a melt curve peak analysis was performed to ensure amplification of only the correct product.

For each gene 6 dilutions of cDNA (equivalent to 100, 50, 25, 12.5, 6.25, 3.12 ng of starting RNA) were run in triplicate to determine PCR efficiency per sample. A threshold was chosen so that amplification was in the exponential phase and the correlation coefficient (as found for the linear relation between threshold cycle value and log of starting quantity) was maximal. The average threshold cycle for triplicate measurements was used to calculate expression ratios. Differences in expression between the exposed samples and the control sample were tested for significance using Mann-Whitney U-test ($P < 0.05$).

Labelling and hybridization

RNA was labeled indirectly using a modification of the aminoallyl labeling method from DeRisi lab (www.microarrays.org). 25 µg of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen Life Technologies, Breda, the Netherlands), in the presence of aminoallyl-dUTP (Sigma, St. Louis, MO, USA) which was built into the cDNA. The reaction mixture was incubated at 42 °C for one hour. After cDNA synthesis, RNA was removed by adding Rnase (15 min,

37 °C) and the resulting cDNA was purified using QIAquick spin columns (Qiagen, Hilden, Germany). The cDNA was dried and resuspended in 5 µl water. To couple Cy3 or Cy5 to the amino-modified cDNA, sodium bicarbonate and NHS-Cy3 or NHS-Cy5 (Amersham Biosciences, Freiburg, Germany) were added to the cDNA and incubated for 1 hour. Uncoupled dye was removed from the solution by purification with QIAquick spin columns. cDNA from cells exposed to 5 or 50 µM quercetin was labeled with Cy3 and cDNA from cells exposed to ethanol (solvent control, 0 µM quercetin) was labeled with Cy5.

Before hybridization, Cy3- and Cy5-labeled cDNAs were mixed and human cot-1 DNA (30 µg, Life Technologies S. A., Merelbeke, Belgium), yeast tRNA (100 µg, Life Technologies S. A., Merelbeke, Belgium) and poly(dA-dT) (20 µg, Amersham Biosciences, Freiburg, Germany) were added to avoid non-specific binding. The hybridization mix was dissolved in 30 µl Easyhyb hybridization buffer (Roche Diagnostics, Mannheim, Germany) and denatured for 1.5 min at 100 °C.

Before adding the hybridization mix to the slides, slides were prehybridized in prehybridization buffer (5 x SSC, 0.1 % SDS and bovine albumine serum (10 mg/ml)) for 2 hours at 42 °C, washed in milliQ water, washed with isopropanol and dried.

After pipetting the hybridization mix on the slides, the slides were covered with a plastic coverslip and hybridized overnight in a slide incubation chamber (Corning, Life Sciences, Schiphol, the Netherlands) submerged in a 42 °C waterbath. After hybridization, slides were washed once by firm shaking in 2 x SSC buffer with 1 % SDS followed by firm shaking in 0.1 x SSC buffer twice, then slides were dried quickly by centrifugation at 700 rpm.

Slides were scanned with a ScanArray 4000 (Perkin Elmer Life Sciences, USA) and Imagene 4.0 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images. For each comparison (5 vs 0 µM quercetin and 50 vs 0 µM quercetin) 4 hybridizations were performed.

Data analysis

Data were transferred from Imagene to Microsoft Excel 97 (Microsoft Corporation, USA) for analysis. First, a threshold was set for signal/background ratio in both channels, based on measured fluorescence of spots without cDNA. About 10–25 % of the spots had fluorescence below this threshold in one or both channels and were excluded from data analysis. Then, local background intensity was subtracted from mean signal intensity and expression ratios were calculated by dividing background corrected signal intensity for Cy3 by background corrected signal intensity for Cy5. Since all cDNAs were present twice on each slide, mean expression ratios for duplo spots were calculated, provided that expression ratios were calculated for both spots. Mean expression ratios were log transformed (base 2) and normalized (for each slide) using an intensity-dependent method (Lowess) [22]. After normalization, data from the 4 duplo slides were combined and for each gene a mean expression ratio for 5 vs 0 µM quercetin and for 50 vs 0 µM quercetin was calculated.

A subset of differentially expressed genes was defined by selecting genes that were on the edges of the data distribution (outliers, identified by correcting mean expression ratios for spot intensity) or by using the SAM tool, based on Tusher et al. [23], to select genes that were in the top 100 for SAM scores. These methods were chosen to ensure a low false discovery rate for the genes identified as differentially expressed. In addition, for all genes in this subset of differentially expressed genes statistical significance was determined using a Student's T-test.

For all selected genes, the accession number was used to search the NCBI Unigene website (www.ncbi.nlm.nih.gov/Unigene/) for the most recent gene names. Information on the genes was obtained from the NCBI websites UniGene, LocusLink, OMIM and PubMed (www.ncbi.nlm.nih.gov). Although the genes on the slides were 'known' genes and not ESTs, of the selected genes about 40 accession numbers were at this time unknown or returned an interim or not official name. Also, for some genes function was unknown.

Results

The expression of most of the genes studied did not change as a result of the treatment with quercetin. After applying the criteria as described in materials and methods, 150–200 differentially expressed genes were selected in the 5 µM quercetin dataset and in the 50 µM quercetin dataset. All selected genes had a mean expression ratio (log transformed, base 2) significantly different from 0 ($P < 0.05$).

Genes were categorized in seven functional groups: 1) cell cycle and cell differentiation (Table 1), 2) apoptosis (Table 2), 3) tumor suppressor genes and oncogenes (Table 3), 4) cell adhesion and cell-cell interaction (Table 4), 5) transcription (Table 5), 6) signal transduction (Table 6) and 7) energy metabolism (Table 7).

Almost all of the differentially expressed genes involved in cell cycle and differentiation (Table 1) were downregulated, specifically in the 5 µM quercetin dataset. For the majority of the genes this would indicate inhibition of cell proliferation. However, PPP2R4 is implicated to play a role in negative control of cell growth and SIAH1 is a mediator of cell cycle arrest, tumor suppression and apoptosis [24]. Genes involved in different

Table 1 Expression changes of genes involved in cell cycle and cell differentiation

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 μM quercetin	50 μM quercetin
H59204	Cdc6 Cell division cycle 6 homolog	CDC6	–4.17*, c	
AA452933	Histone 1, H2ac	HIST1H2AC	–3.93*, d	–2.41
W81318	H1 histone family, member X	H1FX	–3.16*, b	
AA428377	Polymerase (DNA directed), δ2, regulatory subunit (50kD)	POLD2	–3.10*, a	
AA488188	RCD1 required for cell differentiation1 homolog (S. pombe)	RQCD1	–2.88*, c	–2.23*, a
AA460827	Protein phosphatase 1, regulatory (inhibitor) subunit 1A	PPP1R1A	–2.84*, c	–4.06a
AA450062	Prostate differentiation factor	PLAB	–2.42*, a	–2.65*, a
AA074222	Squamous cell carcinoma antigen recognized by T cells	SART1	–2.38*, d	–1.79
T72030	Seven in absentia homolog 1 (Drosophila)	SIAH1	–2.39a	–1.62
AA490617	Vaccinia related kinase 2	VRK2	–2.29*, c	–1.69
R51209	protein phosphatase 2A, regulatory subunit B′	PPP2R4	–2.27*, c	–1.43
H52729	PWP2 periodic tryptophan protein homolog (yeast)	PWP2H	–2.26a	–1.53
AA708161	RNA binding motif, single stranded interacting protein 2	RBMS2	–2.18*, a	–1.37
AA458870	CDC37 cell division cycle 37 homolog (S. cerevisiae)	CDC37	–2.17a	–1.61
AA282936	M-phase phosphoprotein 1	MPHOSPH1	–2.06c	–1.39
AA599145	ZW10 homolog, centromere/kinetochore protein (Drosophila)	ZW10	–2.03	–2.19*, c
AA456291	Developmentally regulated GTP binding protein 2	DRG2	–2.00a	–2.08*, c
R32848	S100 calcium binding protein P	S100P	–1.82b	1.15
AA481076	MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	–1.74*, c	–1.61
N31587	RNA binding motif, single stranded interacting protein 1	RBMS1	–1.69a	–1.60
AA486208	Cyclin-dependent kinase 4	CDK4	–1.63	–2.17b
AA629262	Polo-like kinase (Drosophila)	PLK	–1.55	–1.68*, c
AA707650	Polymerase (DNA directed), α	POLA	–1.52	–2.01*, a
AA487486	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	CCND1	–1.47*, c	–1.17
AA054287	RNA binding motif protein 3	RBM3	–1.37	–2.07*, c
AA488324	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	BUB1B	1.51*, c	1.54*, d
AA009609	Fibroblast growth factor 7 (keratinocyte growth factor)	FGF7	2.18*, d	2.40 *, a

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure. Values < 0 indicate downregulation, values > 0 indicate upregulation
* Indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$

phases of the cell cycle are downregulated, PWP2H has a role in the early G1 phase, CDK4 and cyclin D1 are active during the G1-S phase and M-phase phosphoprotein induces the transition from the G2 to M phase. Also, genes involved in DNA replication (CDC6, POLA, POLD2, RBMS1, RBMS2), in aligning chromosomes (MAD2L1) and in chromosome segregation (ZW10) were downregulated. Two histone family members (HIST1H2AC and H1FX) were markedly downregulated after exposure to 5 μM quercetin. Overexpression of S100P is indicated to be important during tumor progression *in vivo* [25], this gene was downregulated by 5 μM quercetin. A few of the listed genes (RQCD1, PLAB, S100P, FGF7) are involved in cell differentiation rather than regulation of cell cycle progression. Quercetin at 50 μM upregulated FGF7 expression, upregulation of FGF7 could indicate increased cell differentiation [26]. Expression of BUB1B (involved in mitotic checkpoint control) was also upregulated by quercetin.

Table 2 lists genes involved in apoptosis that were differentially expressed in Caco-2 cells in response to quercetin. APAF1, downregulated by 5 μM quercetin, is an important part of the caspase cascade. In response to cytochrome c release from mitochondria APAF1 activates caspase-9, which results in activation of caspase-3. In this study caspase-9 was downregulated by 50 μM quercetin, although this effect did not meet the selection criteria set for differential expression. Transcription of caspase-1 (CASP1) was downregulated by 50 μM quercetin. SERPINB9, also known as PI9, is an inhibitor of CASP1 [27]. SERPINB9 and SERPINB3 were upregulated by both quercetin concentrations, which is consistent with the observed downregulation of CASP1. In cDNA microarray analysis it was found that SFRP1, downregulated by 50 μM quercetin, is methylated in Caco-2 cells and also in most other colon cancer cell lines, resulting in lower expression [28]. Expression of TP53BP2 was upregulated by 5 μM quercetin.

Table 3 lists tumor suppressor genes and oncogenes. Quercetin upregulated expression of two tumor sup-

Table 2 Expression changes of genes involved in apoptosis

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 µM quercetin	50 µM quercetin
N51014	apoptotic protease activating factor	APAF1	–2.22[a]	–1, 47
T95052	caspase 1	CASP1	–1.81	–1.93*,[c]
T68892	secreted frizzled related protein 1	SFRP1	–1.74	–1.82[a]
N48652	Tumor protein p53 binding protein, 2	TP53BP2	1.76*,[c]	1.31
AA398883	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3	SERPINB3	1.73*,[b]	1.65[c]
AA430512	Serine (or cysteine) proteinase, clade B (ovalbumin), member 9	SERPINB9	2.06*,[c]	1.86 *,[c]

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure
Values < 0 indicate downregulation, values > 0 indicate upregulation
* Indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$

Table 3 Expression changes of tumor suppressor genes and oncogenes

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 µM quercetin	50 µM quercetin
H48122	Breast cancer 2, early onset	BRCA2	1.86[a]	1.73[b]
AA857748	Mucin 2, intestinal/tracheal	MUC2	1.78*,[a]	2.23*,[c]
AA670215	Tumor susceptibility gene 101	TSG101	1.68[b]	1.65
AA486280	Tissue inhibitor of metalloproteinase 2	TIMP2	1.53	1.79*,[c]
AA464600	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.46	2.01*,[c]
AA130584	Carcinoembryonic antigen-related cell adhesion molecule 5	CEACAM5	–3.04*,[b]	1.51
H94892	V-ral simian leukemia viral oncogene homolog A (ras related)	RALA	–2.10[a]	–1.80[c]

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure
Values < 0 indicate downregulation, values > 0 indicate upregulation
* Indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$

pressor genes, BRCA2 and MUC2. BRCA2 is reported to have a role in DNA repair and thus to maintain chromosomal stability [29]. Recently, it was concluded in a study with MUC2–/– mice that MUC2 suppresses colorectal cancer [30]. TSG101 is involved in growth and differentiation control in epithelial cells [31]. TIMP2 is known as a metastasis suppressor. TSG101 and TIMP2 are also upregulated. CEACAM5 (at 5 µM) and RALA (at 5 and 50 µM) are downregulated by quercetin. RALA is a potential stimulator of tumor metastasis [32]. Expression of the proto-oncogene MYC was upregulated after exposure to 50 µM quercetin.

Downregulation of genes involved in cell adhesion and cell-cell interaction was found at both quercetin concentrations (Table 4). Plakophilin 2 (downregulated at 5 and 50 µM quercetin) has a function in intercellular junctions. Overexpression of AOC3 was found in gastric cancer [33], in this study AOC3 was downregulated by 5 µM quercetin. Several other cell adhesion genes were upregulated, especially at the higher quercetin concentration (50 µM). Among these genes are two cadherins (2 and 11) and alpha-catenin. Alpha-catenin was upregulated at both quercetin concentrations. Downregulation of alpha-catenin in colorectal cancer is a marker for poor differentiation and high metastatic potential [34]. Cadherin 2, which also was upregulated by quercetin, has been found to promote motility and invasion in breast cancer cells [35]. Thrombospondin 2 can act as a angioinhibiting factor and contribute to inhibition of colon cancer metastasis [36].

Genes involved in transcription that were differentially expressed in response to quercetin are shown in Table 5. PAX8 has been reported to activate the BCL2-promoter and to inhibit the p53-promotor, thus regulating expression of these genes [37, 38]. RELB is a member of the NF-kappaB family and can regulate transcription of p21 [39]. HnRNP proteins (HNRPDL and HNRPA0) are involved in mRNA processing, metabolism and transport. PAX8, RELB and two hnRNP genes were all downregulated by 5 µM quercetin. Many effects have

Table 4 Expression changes of genes involved in cell adhesion and cell-cell interaction

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 µM quercetin	50 µM quercetin
AA485353	Lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	–3.45*, b	–2.99*, d
AA036975	Amine oxidase, copper containing 3 (vascular adhesion protein 1)	AOC3	–2.97*, c	–2.30
AA292676	A disintegrin and metalloproteinase domain 15 (metargidin)	ADAM15	–2.63*, b	–1.54
H17975	Armadillo repeat gene deletes in velocardiofacial syndrome	ARVCF	–2.37*, c	–1.43
H66158	plakophilin 2	PKP2	–2.20a	–2.14*, c
H56349	Fibrinogen-like 2	FGL2	–2.03*, a	–1.48
AA457739	Claudin 10	CLDN10	–1.69	–1.81*, c
H54417	Non-metastatic cells 4, protein expressed in	NME4	–1.44	–1.71*, c
T46897	Adhesion regulating molecule 1	ADRM1		–1.69*, c
AA489587	Fibronectin 1	FN1	–1.40c	1.39
AA190508	A disintegrin and metalloproteinase domain 12 (meltrin alpha)	ADAM12	1.61*, c	1.25
AA676957	Catenin (cadherin-associated protein), a1 (102kD)	CTNNA1	1.63b	1.56a
AA400329	Neurofilament 3 (150kD medium)	NEF3	1.62*, c	1.69a
AA452840	Fibulin 2	FBLN2	1.53	1.77*, c
R79948	Formyl peptide receptor-like 1	FPRL1	1.42	1.82*, a
R43483	Integrin, alpha 6	ITGA6	1.65*, c	
W49619	Cadherin 2, N-cadherin (neuronal)	CDH2	1.92a	1.85a
AA136983	Cadherin 11 (OB-cadherin)	CDH11	1.80	1.91*, a
AA133469	Cytokeratin 20	KRT20	1.96	1.93*, c
AA875933	EGF-containing fibulin-like extracellular matrix protein 1	EFEMP1	1.85	2.09*, c
H24006	Oligodendrocyte myelin glycoprotein	OMG	2.13*, a	2.46*, a
R56774	Bone morphogenetic protein 1	BMP1	2.46	2.08a
H38240	Thrombospondin 2	THBS2	2.25	2.45*, a

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For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$

been described for EGR1, ranging from growth stimulation to growth suppression and from anti-apoptotic to pro-apoptotic [40]. EGR1 can interact with nuclear transcription factor and tumor suppressor p53 [41]. EGR1 was downregulated by 50 µM quercetin. STAT1, downregulated by 50 µM quercetin, is involved in regulation of cell cycle and apoptosis [42]. MITF interacts with LEF-1, a member of the Wnt-signalling pathway [43] and can be a target of the p38 MAPK pathway [44]. MITF was upregulated by quercetin.

Three members of the MAPK signal transduction pathway were downregulated by quercetin, MAP4K1, MAP3K12 and MAP2K4 (Table 6). These MAP kinases can activate JNK1, JNK2 and p38. Other downregulated signal transduction genes include MADH5, SIAH2 and IFNGR2. MADH5 is a member of the SMAD protein family, which is involved in transduction of signals from the TGF-beta pathway. The SIAH pathway is reported to influence TGF-beta/SMAD signaling [45]. Increased expression of IFN-gamma receptors, associated with increased IFN-gamma responsiveness, was reported earlier in post-confluent Caco-2 cells [46]. Several G-protein coupled receptors were upregulated, like VIPR1 and EDNRB (at 5 µM) and P2RY2 (at 5 and 50 µM), and in addition several regulators of G-protein signaling were differentially expressed (RGS7, RGS1 and RGS19IP1). Two interleukin receptors (IL1R1 and IL4R) were downregulated by 5 µM quercetin. Two members of the tumor necrosis factor receptor superfamily (TNFRSF1B and TNFRSF11B) were upregulated by quercetin. Interestingly, after exposure to 50 µM quercetin expression of a protein kinase C isoform (PRKCQ) was upregulated.

Downregulation of genes involved in energy metabolism was found predominantly after exposure to 50 µM quercetin (Table 7). GALNT1 is involved in glycosylation. It was recently found that high expression of GALNT1 in colorectal carcinomas is associated with a better prognosis [47]. Two vacuolar ATPases (ATP6V1C1 and ATP6V0B) were also downregulated. Vacuolar ATPases could have anti-apoptotic effects in human cancer cells [48]. Interestingly, PDK2 is downregulated, but PDK4 is upregulated after exposure to 50 µM quercetin.

Table 5 Expression changes of genes involved in transcription

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 μM quercetin	50 μM quercetin
AA490538	Zinc finger protein 161 homolog (mouse)	ZFP161	–3.40*, c	–2.46*, a
AA405767	Paired box gene 8	PAX8	–3.42a	–1.81
AA489785	Nuclear receptor coactivator 1	NCOA1	–3.18*, b	–2.66
AA258001	Transcription factor RELB	RELB	–3.18*, b	–3.02*
AA598578	Heterogeneous nuclear ribonucleoprotein D-like	HNRPDL	–2.39*, b	–1.72
AA419238	Retinoic acid receptor, β	RARB	–2.20a	–1.73
AA432143	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1	CITED1	–2.04*, a	–2.53*, a
N77807	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)	MLL	–1.91	–2.13*, c
AA599176	Heterogeneous nuclear ribonucleoprotein A0	HNRPA0	–1.87a	–1.95
AA488075	Signal transducer and activator of transcription 1	STAT1	–1.36	–1.70*, a
AA630017	Transcription elongation factor B (SIII), polypeptide 2 (18kD, elongin B)	TCEB2	–1.35	–1.78b
AA486533	Early growth response protein 1	EGR1		–2.07*, d
AA099534	Activated RNA polymerase II transcription cofactor 4	PC4	1.59a	1.39
H91651	GA-binding protein transcription factor, beta subunit 2 (47kD)	GABPB2	1.62	2.12*, d
AA150301	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32 kD	TAF9	1.64c	1.47
W96099	Retinoid X receptor-γ	RXRG	1.66	1.78*, c
W47015	Ts translation elongation factor, mitochondrial	TSFM	1.75c	1.79b
T64905	Paired-like homeodomain transcription factor 2	PITX2	1.87	2.09*, a
AA102068	Heat shock transcription factor 4	HSF4	1.90*, a	1.60
AA454222	Bromodomain, testis-specific	BRDT	2.10	2.71*, b
N66177	Microphthalmia-associated transcription factor	MITF	2.18*, c	2.49*, c

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure
Values < 0 indicate downregulation, values > 0 indicate upregulation
* Indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$

Of all genes studied, CYP1A1 was the gene most induced by quercetin in Caco-2 cells (10.6-fold increase after exposure to 50 μM quercetin). No induction of CYP1A1 was found after exposure to 5 μM quercetin. In breast cancer cells quercetin also caused a time- and concentration-dependent increase in CYP1A1 mRNA levels [49]. Several membrane proteins were differentially expressed in response to quercetin (data not shown). Three potassium channel genes were downregulated, either after exposure to 5 μM quercetin (KCNK3 and KCNQ2) or after exposure to 50 μM quercetin (KCNAB1). Expression of two other potassium channels was upregulated, KCNMB1 after exposure to 5 μM quercetin and KCNA1 after exposure to 50 μM quercetin. In addition, four members of the solute carrier family were downregulated, SLC2A3 and SLC14A1 after exposure to 5 μM quercetin and SLC1A3 and SLC6A8 after exposure to 50 μM quercetin. The transporter gene ABCC3, also known as MRP3, was upregulated after exposure to 50 μM quercetin. Induction of MRP3 gene expression was also found in two human colon cancer cell lines after exposure to a NSAID [50].

Expression of CDK4, RALA and CYP1A1 was also measured by real-time RT-PCR to verify the expression alterations measured by microarray analysis. Downregulation of expression of CDK4 by quercetin was confirmed by real-time RT-PCR experiments (Fig. 1a). Real-time PCR showed a stronger downregulation of CDK4 by 5 μM quercetin than the weak non-significant downregulation found in the microarray experiment. Downregulation of expression of RALA was also found in the real-time RT-PCR experiments (Fig. 1b). However, when expression of RALA was normalized using GAPDH expression, downregulation of RALA by 50 μM quercetin was not significant. Upregulation of CYP1A1 gene expression was confirmed by real-time RT-PCR. Interestingly, the induction of CYP1A1 gene expression by 50 μM quercetin, which was ~10-fold in the microarray experiment, was much higher in the real-time RT-PCR experiment (~600-fold when normalized to beta-actin and ~800-fold when normalized to GAPDH, data not shown).

The gene expression data indicate a marked effect of quercetin on cell cycle. This was studied in more detail. After 48 hours exposure to quercetin, no cytotoxicity was observed in Caco-2 cells at either concentration. Exposure to 5 μM quercetin resulted in a significant decrease in cell proliferation (87.4 ± 7.7 % of control;

Table 6 Expression changes of genes involved in signal transduction

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 μM quercetin	50 μM quercetin
AA053674	Mitogen-activated protein kinase kinase kinase 12	MAP3K12	−2.42[a]	−1.74[a]
H05140	Regucalcin (senescence marker protein-30)	RGN	−2.02*, [c]	−3.31
T50313	Mitogen-activated protein kinase kinase kinase kinase 1	MAP4K1		−2.77[a]
H24326	Regulator of G-protein signalling 7	RGS7	−2.01*, [a]	−2.46
AA293365	Mitogen-activated protein kinase kinase 4	MAP2K4	−2.02*, [c]	−2.39*, [c]
R24266	Growth factor receptor-bound protein 14	GRB14		−2.26*, [c]
AA424700	MAD, mothers against decapentaplegic homolog 5 (Drosophila)	MADH5	−2.10[a]	−2.14*, [c]
AA029042	Seven in absentia homolog 2 (Drosophila)	SIAH2	−2.14*, [b]	−2.11*, [b]
AA284492	Tetraspan 3	TSPAN-3	−1.70	−2.03*, [d]
AA449440	Interferon gamma receptor 2 (interferon gamma transducer 1)	IFNGR2	−2.59	−1.91*, [c]
AA810225	G protein-coupled receptor 30	GPR30	−2.02	−1.88*, [a]
AA464526	Interleukin 1 receptor, type 1	IL1R1	−2.03[a]	−1.62
H29322	Calcium/calmodulin-dependent protein kinase I	CAMK1	−1.62	−1.54[a]
AA293306	Interleukin 4 receptor	IL4R	−1.89*, [a]	−1.27
AA017544	Regulator of G-protein signalling 1	RGS1	1.66	1.87*, [c]
AA779480	Bone morphogenetic protein 8 (osteogenic protein 2)	BMP8	1.73[b]	1.39
H73241	Vasoactive intestinal peptide receptor 1	VIPR1	1.75*, [c]	1.49
AA150507	Interleukin 1, beta	IL1B	1.86*, [a]	1.65
H28710	Endothelin receptor type B	EDNRB	2.18[a]	1.72*, [c]
R68106	Fc fragment of IgG, low affinity IIb, receptor for (CD32)	FCGR2B	1.87*, [c]	1.75[b]
R70505	Purinergic receptor P2Y, G-protein coupled, 2	P2RY2	2.00*, [c]	1.86[a]
AA102526	Interleukin 8	IL8	1.38	1.88*, [c]
R48132	SH3-domain binding protein 2	SH3BP2	1.70*, [c]	1.97[a]
H60824	Protein kinase C, theta	PRKCQ	1.78	1.99*, [c]
T99303	Guanine nucleotide binding protein (G protein), α15 (Gq class)	GNA15	1.95*, [a]	2.04*, [c]
AA434159	regulator of G-protein signalling 19 interacting protein 1	RGS19IP1	1.86	2.20*, [b]
AA421269	Phosphatidylinositol 4-kinase, catalytic, α-polypeptide	PIK4CA	2.13*	2.33*, [d]
AA150416	Tumor necrosis factor receptor superfamily, member 1B	TNFRSF1B	1.96	2.42*, [c]
AA194983	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	TNFRSF11B	2.02*, [b]	1.93[a]
N53351	Ras-like without CAAX 2	RIT2	2.32*, [b]	

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure
Values < 0 indicate downregulation, values > 0 indicate upregulation
* indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$; [d] $P < 0.001$

$P < 0.05$). Exposure to 50 μM quercetin however resulted in a much larger decrease in cell proliferation to 51.3 ± 5.1 % of control ($P < 0.001$). Inhibition of cell proliferation of Caco-2 cells by quercetin showed a biphasic response, at low quercetin concentrations (0.5–5 μM) cell proliferation was decreased to about 90 % of control and at concentrations higher than 10 μM cell proliferation showed a more profound dose-dependent decrease (Fig. 2). Cell cycle analysis showed a decrease in the percentage of cells in the G1-phase and an increase in the percentage of cells in the S-phase when cells exposed to 5 μM quercetin were compared to control (Fig. 3). After exposure to 50 μM quercetin the percentage of cells in the G1-phase further decreased and the number of cells in the sub-G1 phase increased (Fig. 3, $P < 0.05$ compared to 5 μM quercetin). This indicates that the decrease in cell proliferation after exposure to 5 μM quercetin could be due to S-phase arrest and that the additional decrease in cell proliferation after exposure to 50 μM quercetin could be due to a shift from cells in the G1-phase to the sub-G1 phase.

Discussion

This study describes the effects of quercetin on the expression of 4000 human genes in Caco-2 cells. An interesting observation was the downregulation of many cell cycle genes after exposure to 5 μM quercetin. It was shown that this correlated with a modest but significant decrease in cell proliferation. In addition, flow cytometric analyses showed a decrease in the percentage of cells

Table 7 Expression changes of genes involved in energy metabolism

Accession number	Gene name	Gene symbol	Fold up-/down-regulation	
			5 µM quercetin	50 µM quercetin
AA680322	NADH dehydrogenase (ubiquinone) 1 α subcomplex, 4 (9kD, MLRQ)	NDUFA4	–2.94*, a	–4.93
AA706987	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GalNAc-T1)	GALNT1	–1.88	–2.94*, a
AA485376	Adenosine monophosphate deaminase 2 (isoform L)	AMPD2	–2.05	–2.82[a]
H29475	Pyruvate dehydrogenase kinase, isoenzyme 2	PDK2		–2.45*, a
H05768	ATPase, H+ transporting, lysosomal 42kD, V1 subunit C, isoform 1	ATP6V1C1	–1.51	–2.05[a]
AA062805	Succinate dehydrogenase complex, subunit C	SDHC	–1.70	–1.71*, c
AA457717	ATPase, H+ transporting, lysosomal 21kD, V0 subunit c	ATP6V0B	–1.25	–1.54[b]
H08732	Glycogen synthase 1 (muscle)	GYS1	1.42*, d	1.62[b]
AA169469	Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	2.12	1.99*, c

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure
Values < 0 indicate downregulation, values > 0 indicate upregulation
* Indicates differential expression according to SAM analysis
For all genes identified as differentially expressed a P-value was calculated using a t-test
[a] $P < 0.05$; [b] $P < 0.02$; [c] $P < 0.01$

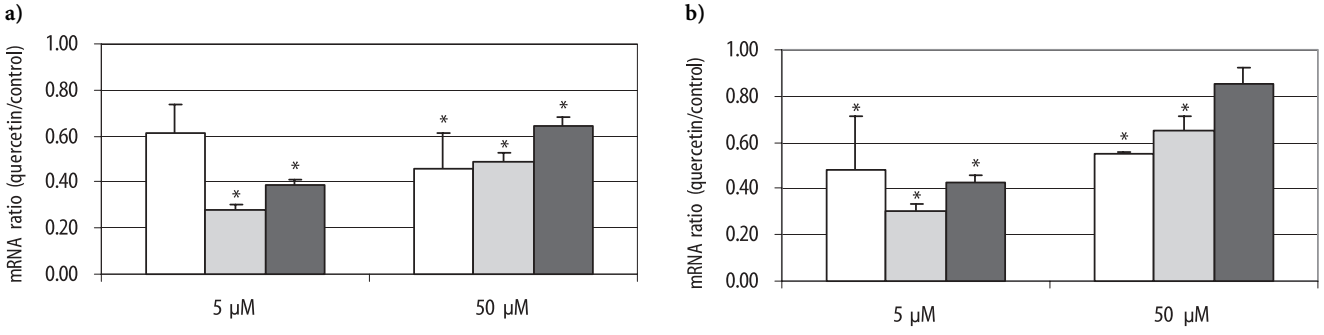

Fig. 1 **a** CDK4 gene expression changes in response to quercetin, measured with cDNA microarray (white bars), and measured by real time RT-PCR normalized to beta-actin expression (grey bars) or to GAPDH expression (black bars). Normalized expression is plotted as mRNA ratio (quercetin/control). Values are mean ± standard deviation (n = 5). * expression is significantly different from control ($P < 0.05$). **b** RALA gene expression changes in response to quercetin, measured with cDNA microarray (white bars), and measured by real time RT-PCR normalized to beta-actin expression (grey bars) or to GAPDH expression (black bars). Normalized expression is plotted as mRNA ratio (quercetin/control). Values are mean ± standard deviation (n = 5). * expression is significantly different from control ($P < 0.05$)

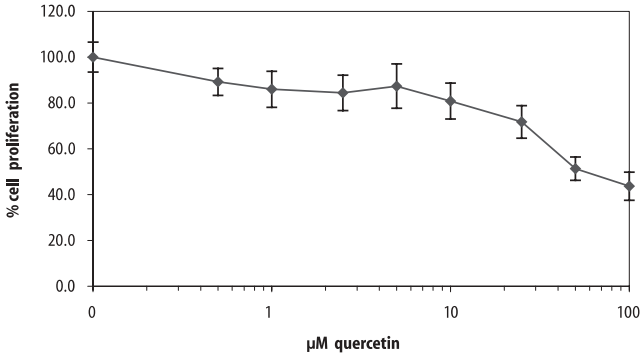

Fig. 2 Cell proliferation of Caco-2 cells exposed to quercetin for 48 hours, with medium refreshment after 24 hours, measured with BrdU incorporation. Proliferation in cells exposed to solvent control was set to 100 %, values are mean ± standard deviation (n = 8)

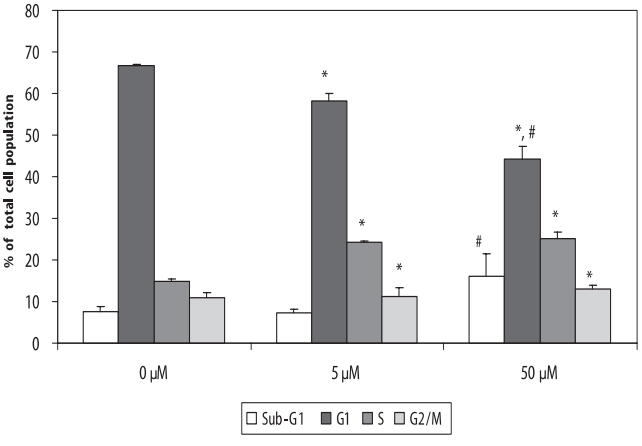

Fig. 3 Cell cycle distribution of Caco-2 cells exposed to quercetin for 48 hours, with medium refreshment after 24 hours, measured with propidium iodide assay. Values are mean ± standard deviation (n = 4). * significantly different from 0 µM, $P < 0.05$; # significantly different from 5 µM, $P < 0.05$

in the G1 phase and an increase in the percentage of cells in the S-phase. This coincided with a downregulation of CDK4 and cyclin D1 expression by 5 μM quercetin. Similar results were reported for resveratrol; this plant polyphenol decreased cell proliferation of Caco-2 cells, caused an S-phase arrest and downregulated protein expression of CDK4 and cyclin D1 dose-dependently [51]. The CDK4/cyclin D complex is active at the transition from the G1- to the S-phase. Expression of cyclin D1 and CDK4 was found to be higher in adenomas than in normal colon mucosa in AOM-treated mice [52]. In addition, cyclin dependent kinase inhibitor p27 (CDKN1B) was upregulated after exposure to 5 μM quercetin, although this effect did not meet the selection criteria set for differential expression. Upregulation of p27 could have a role in blocking progression through the S-phase [53]. CDC6 has a role in DNA replication during the S-phase [54], so the downregulation of this gene by quercetin could also be related to the S-phase arrest. Downregulation of CDC6 gene expression was also reported in human prostate cancer cells after exposure to genistein, a soy isoflavone [55]. In some colon cancer cell lines quercetin caused a block in G0/G1 phase [56], in others in G2/M phase [57]. Chalcone, a precursor of flavonoids, was reported to inhibit transition from the S to the G2/M phase in colon cancer cells [57].

Exposure of Caco-2 cells to 50 μM quercetin resulted in a larger decrease in cell proliferation than exposure to 5 μM quercetin (51.3 % of control vs 87.4 % of control). The percentage of cells in the G1 phase showed an additional decrease compared to cells exposed to 5 μM quercetin, at the same time the percentage of cells in the sub-G1 phase increased. This indicates that the inhibition of cell proliferation by 50 μM quercetin could partly be attributed to an increase in the number of apoptotic cells in the sub-G1 phase. Downregulation of PLK in SW480 colon cancer cells did not only inhibit cell proliferation, but also induced apoptosis [58]. This suggests that downregulation of PLK by 50 μM quercetin could be related to the increase of percentage of cells in the sub-G1 phase. Similarly, genes like tumor suppressor genes or other cell cycle related genes could also be involved in the process of apoptosis, in addition to differentially expressed genes involved in apoptosis shown in Table 2. For example TP53BP2, listed in Table 2, has been reported to be involved in both apoptosis [59] and cell cycle arrest [60], and could be involved in the tumor suppressing activity of p53. Also, CEACAM5, listed in Table 3 (tumor suppressor genes and oncogenes), was found to be anti-apoptotic in HT29 cells [61]. Furthermore, the level of APAF1 function can be regulated both at transcriptional and post-translational level [62], which is an example of the fact that the process of apoptosis is for a large part regulated at the protein level, for example by activation of procaspases [63]. In addition, it is possible that effects on apoptotic genes are more specifically found at earlier time points. At concentrations higher than those used in this study, quercetin was able to induce apoptosis in human colon cancer cells. Caco-2 cells exposed to 100 μM quercetin for 3 days showed chromatin condensation, indicating apoptosis [12]. In another human colon cancer cell line 200 μM quercetin caused DNA fragmentation [11]. In breast cancer cells, apoptosis was increased by lower concentrations of quercetin (20 μM) after exposure of 24 hours or longer [64]. Expression of MYC, known as an oncogene, was upregulated by 50 μM quercetin. However, in Caco-2 cells exposed to the non-steroidal anti-inflammatory drug (NSAID) indomethacin downregulation of cell proliferation and induction of apoptosis coincided with an increase in expression of c-myc, p53 and p27 [65]. The differentiated action of quercetin on the Caco-2 cell line (subtle effects on cell cycle through gene regulation and more severe apoptotic/cytotoxic effects at higher concentrations) as observed in the gene expression data and flow cytometry, were also confirmed in the *in vitro* cell proliferation data, where a biphasic decrease in cell proliferation was observed.

Recently, changes in gene expression profile during differentiation of Caco-2 cells were described [66]. Some of the genes differentially expressed in response to quercetin showed similar expression changes during Caco-2 differentiation, for example CDC6 (downregulated), cyclin D1 (downregulated), DNA polymerase alpha and delta (downregulated), M phase phosphoprotein 1 (downregulated), hnRNP genes (downregulated), ABCC3 (upregulated), BMP1 (upregulated), fibronectin 1 (downregulated). This was more pronounced at the lower quercetin concentration, indicating that quercetin possibly can promote differentiation of Caco-2 cells.

These effects of quercetin on cell cycle and differentiation, together with the effect on expression of tumor suppressor genes and metastasis suppressors, support the anticarcinogenic potential of quercetin in Caco-2 cells. Another mechanism of anti-carcinogenicity could be increased cell-cell interaction. Both up- and downregulation of genes involved in cell adhesion and cell-cell interaction was found in Caco-2 cells after exposure to quercetin. Upregulation of alpha-catenin could be an important mechanism of anti-carcinogenicity since alpha-catenin can act as an invasion suppressor [67]. Alpha-catenin expression in colon cancer cells is associated with an inhibition of TCF-dependent transcription [68]. Also, overexpression of plakophilin 2 in SW480 cells (human colon carcinoma cell line) caused an increase in β-catenin/TCF signaling [69]. This indicates that upregulation of alpha-catenin expression and downregulation of plakophilin 2 expression could result in decreased beta-catenin/TCF signaling. The beta-catenin/TCF signaling pathway, which is a part of the Wnt signaling cascade, is often deregulated in colon cancer [70]. SFRP1, downregulated by 50 μM quercetin, can

also influence the Wnt signaling pathway [71]. Other genes that were differentially expressed in response to quercetin and that are involved in beta-catenin/TCF signaling include MITF and SIAH1. Quercetin influenced more signal transduction pathways at the gene expression level. Several genes involved in TGF-beta/SMAD signal transduction (BMP1, BMP8, MADH5) and in MAPK signal transduction (MAP2K4, MAP3K12 and MAP4K1) were differentially expressed.

Although at protein level quercetin and other flavonoids are known as potent protein kinase C (PKC) inhibitors [72], quercetin upregulated gene expression of a protein kinase C isoform in Caco-2 cells. Upregulation of PKC increased cell-cell adhesion in alpha-catenin negative human colon cancer cells [73]. Also, in another human colon cancer cell line (HT29) overexpression of PKC isoform resulted in inhibition of growth and reduced tumorigenicity [74]. This indicates that PKC could act as a growth-suppressor in human colon cancer cells.

So, in addition to the effect on cell cycle, other leads obtained by this multiple gene expression experiment to study in more detail include the effect of quercetin on beta catenin/TCF signaling since this is an important pathway in colon carcinogenesis. Another interesting lead could be transcription factors influenced by quercetin. In this study, several transcription factors with target genes that are known to play a role in carcinogenesis were differentially expressed in response to quercetin.

Some genes that were differentially expressed are indicated to be involved in processes or tissues that are not yet known to be relevant to in vitro cultured colon cancer cells. On the one hand, this indicates the partly dedifferentiated character of Caco-2 cells. On the other hand, such findings stress the importance of data evaluation and call for care in too straightforward biological interpretation of these microarray data. Therefore, apart from the careful selection of differentially expressed genes applied to the data, also pathway-related evaluation was applied and found to be an effective tool in microarray data analysis. Although *in vitro* systems by their very nature are limited in their comparison to the in vivo situation, it is clear that gene expression studies using microarrays in combination with functional studies of cell proliferation and apoptosis can yield a broad range of interesting information on the mechanisms of food components in intestinal carcinogenesis.

Several studies report physiological effects of quercetin in cultured colon cancer cells after an exposure period of 48 hours [12, 13]. A similar exposure time was chosen in this study, however after 24 hours fresh medium with quercetin was added, to mimic repeated exposure in the in vivo situation. In future studies, it would be interesting to measure expression changes at more and earlier time points, also because gene expression changes can occur already after short-term exposure. By studying time-dependent changes in expression, information on effects of quercetin can be further extended.

In conclusion, this study shows the broad range of effects at gene expression level that quercetin exerts on colon cancer cells in vitro. Differential expression of many cell cycle genes together with the cell cycle arrest in response to quercetin indicate that quercetin specifically influences cell proliferation in Caco-2 cells at a low concentration. Additionally, differential expression of the genes involved in tumor suppression, cell adhesion, transcription and signal transduction were found. All these processes can contribute to the anticarcinogenic potential of quercetin. In this study all selected differentially expressed genes were studied individually and grouped according to function of the gene. Each functional group corresponds to one or more pathways. Using this approach, 'new' genes were found to be differentially expressed in response to quercetin and by grouping these genes an indication of pathways involved in the actions of quercetin was presented.

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