Role of phytochemicals in colon cancer prevention: a nutrigenomics approach

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ABSTRACT

**Role of phytochemicals in colon cancer prevention:
a nutrigenomics approach**

Specific food compounds, especially from fruits and vegetables, may protect against development of colon cancer. In this thesis effects and mechanisms of various phytochemicals in relation to colon cancer prevention were studied through application of large-scale gene expression profiling. Expression measurement of thousands of genes can yield a more complete and in-depth insight into the mode of action of the compounds.
Effects of quercetin (a flavonoid present in e.g. apples and onions), curcumin (a spice used e.g. in curries) and resveratrol (present e.g. in grapes) were studied in cultured colon cancer cells. These studies confirmed some hypothesized mechanisms of action of these compounds (e.g. effects on cell cycle) and yielded new interesting leads (e.g. effects on proteasome genes, DNA repair genes, tubulin genes). In addition, expression profiles of a panel of 14 human cell lines derived from colonic tissue were compared and related to expression profiles of human colon biopsies from normal and tumor tissue. Changes in expression profiles of a subset of colon cancer-specific genes (as a biomarker set) in cultured colon cancer cells could be useful to translate in vitro results to the in vivo situation.
In addition to the in vitro studies, effects of wheat bran, curcumin, rutin and benzyl isothiocyanate on colon carcinogenesis were studied in a rat model. Wheat bran and curcumin showed a protective effect (lower tumor multiplicity after 8 months compared to the control group). Expression profiles of differentially expressed genes in small intestinal tissue at intermediate time points were predictive of colon tumor development at the end of the study, confirming a correlation between effects in small intestine and colon. In summary, the studies in this thesis demonstrate the potential of large-scale expression profiling in nutrition studies. These studies also demonstrated that although the technological advancements in large-scale gene expression analysis allow for the collection of 'whole genome' results, the challenge for the coming years will be to further exploit these data. Only when advances in the bioinformatics field lead to easier interpretation of large amounts of data from nutrigenomics studies, the large potential of nutrigenomics can become reality.

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General Introduction

Based on:
Nutrigenomics and gastrointestinal health promotion
Marjan J van Erk and Ben van Ommen
In: ‘Nutrigenomics and Proteomics in Health Promotion and Disease Prevention’
(to be published in 2005)

Background and aim of this thesis

Development of colon cancer, a common form of cancer in Western countries, is related to diet and dietary compounds. Specifically, epidemiological studies indicate a protective effect of fruit and vegetable consumption. Although many bioactive phytochemicals (compounds from plants) have been identified, little is known about the mechanisms of cancer prevention of these compounds.

At the same time that the human genome sequence was being unraveled, techniques were developed to measure expression of thousands of genes or proteins simultaneously. This advance from studying single genes or proteins to studying thousands of genes or proteins in one experiment promises to yield extensive and more comprehensive information in many areas of research, including nutrition-related research (figure 1.1).

The aim of this thesis is to obtain more complete and in-depth insight in the mode of action of certain phytochemicals in relation to colon cancer prevention through application of large-scale gene expression measurement techniques. This approach has the potential to generate a wealth of information on effects of these compounds, which will help to elucidate the mechanisms of action. The studies described in this thesis are focused on quercetin, rutin, curcumin, resveratrol, wheat bran and benzyl isothiocyanate: plant compounds that are known to be able to protect against colon cancer development.

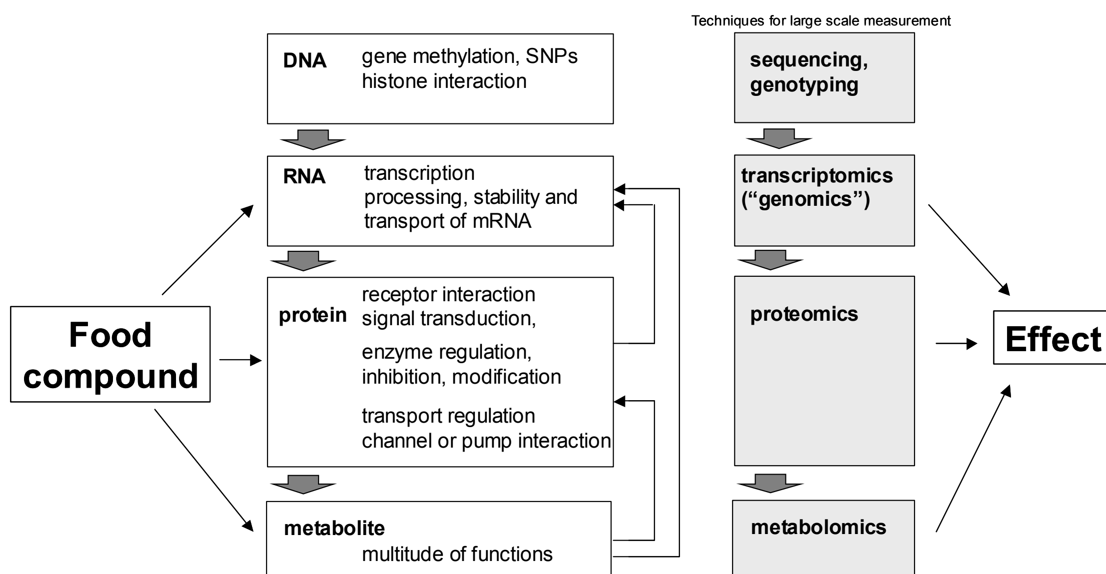

Figure 1.1. Food compounds can have an effect on different levels in the cells (DNA, RNA, protein, metabolite). Large-scale measurement is possible at each of these levels.

Colon cancer

Introduction

Colon cancer is one of the most prevalent forms of cancer in the Western countries. In the Netherlands for example it is the most common form of cancer in males after prostate and lung cancer and in females it is the most common form of cancer after breast cancer [1]. In the USA the incidence of colon cancer was 62.5 cases per 100.000 for males and 45.9 cases per 100.000 for females in the year 2000 (Surveillance, Epidemiology, and End Results (SEER) Program; www.seer.cancer.gov). In the Netherlands, the colon cancer incidence was reported to be 32.8 per 100.000 person-years for males and 26 per 100.000 person-years for females in 1998 [2].

The process of cancer development can be divided into different steps. It starts with DNA damage and mutations in the initiation phase, followed by growth of transformed cells in the promotion stage, leading to malignant growth and invasion in the progression stage. During development of colon cancer normal colonic epithelium transfers into hyperproliferative epithelium and then further on into adenoma, carcinoma and eventually metastasis, through accumulation of genetic alterations, according to the model of Fearon and Vogelstein [3]. Crucial genes involved in this process include APC, K-ras, DCC, p53, c-myc, cox-2, mismatch repair genes, cell adhesion genes [4-6]. Accumulation of alterations during carcinogenesis leads to impairment of normal growth inhibition by increased cell growth and by inhibition of apoptosis, resulting in clonal expansion of tumor cells [7]. Normally, the balance between cell proliferation and apoptosis in colonic mucosa is tightly regulated in order to maintain a constant cell number [8]. The disturbance of the balance results in an escape from the normal homeostasis of cell number and favors the survival of the mutated and undifferentiated cells [9, 10]. Inhibition of proliferation and increase in apoptosis of these aberrant cells are important mechanisms of prevention of colon cancer. Also, promotion of differentiation e.g. by butyrate is an important preventive mechanism, since differentiation makes an escape from the tight control on cell growth less likely.

Several forms of hereditary colon cancer are known. In Familial Adenomatous Polyposis (FAP) patients inherit a germ-line mutation in APC and develop many colon tumors. Hereditary Non-Polyposis Colorectal Cancer (HNPCC) is related to microsatellite instability and a defect in DNA repair [4-6].

Role of nutrition in colon carcinogenesis

Development of cancer is strongly related to environmental factors. Diet and dietary factors are important in different types of cancer, especially cancers in the gastro-intestinal tract. This is clearly shown in studies of migrants, for example changes in the diet of Mexican-Americans living in the USA coincided with an increase in colorectal cancer incidence [11].

Willett estimated that overall between 20 and 42 percent of cancer could be avoided by dietary changes. For colon cancer this could be as high as 50 to 80 percent [12].
Much attention has been given to the possible protective effect of vegetables against development of colorectal cancer. Results of over 200 human epidemiological studies are summarized in a review by Steinmetz and Potter [13]. The majority of the studies on fruit and vegetables found a protective effect of fruits and vegetables against colon cancer, specifically for raw vegetables and green vegetables [13, 14]. A large set of compounds present in fruits and vegetables could contribute to this protective effect, for example flavonoids, folate, isothiocyanates, allium compounds, carotenoids, vitamins, fiber, selenium, etcetera [13]. Also, plant compounds with NSAID-like activity like curcumin are interesting, since use of non-steroidal anti-inflammatory drugs (NSAIDs) including aspirin is strongly associated with reduced risk of colorectal cancer [14]. On the other hand, high intake of fat and meat is associated with higher risk of colon cancer. Production of heterocyclic amines during cooking of meat could contribute to the increased risk with higher meat consumption [14].
Dietary compounds can exert their preventive action at various stages of the process of development of colon cancer. Examples are protection against DNA damage by inhibition of uptake or activation of carcinogens (initiation phase), inhibition of cell proliferation, induction of apoptosis, modulation of signal transduction (promotion phase) and suppression of invasion by inhibition of angiogenesis or by an effect on cell adhesion molecules (progression phase) [15]. Mechanistic studies have pointed out that indeed cancer-preventive food compounds can inhibit cell proliferation, induce apoptosis, modify DNA repair, modify activity of xenobiotic-metabolizing enzymes, modify activity of other enzymes, act as an antioxidant and more. These effects can be mediated through several signaling pathways and transcription factors, including MAPK signal transduction and NFκB and AP1 transcription factors [16-18]. Examples of potential cancer-preventive food compounds and their possible mechanisms of action are shown in table 1.1.

Table 1.1. Food compounds with cancer-preventive properties, the food sources and possible mechanisms of chemoprevention.

Food compound	Major food source	Possible mechanism(s)*								
		1	2	3	4	5	6	7	8	9
carotenoids / vitamin A	fruits, vegetables, cereal					X		X	X	X
chlorophyllin	leafy vegetables				X	X		X		
coumarins	vegetables, citrus fruits					X				
diallyl sulphides	onion, garlic					X				
dietary fiber	fruits, vegetables, seeds	X								
flavonoids	fruits, vegetables, tea		X		X	X	X	X	X	X
folate (vitamin B-complex)	vegetables, liver, cereals			X						
glucosinolates, indoles, isothiocyanates	cruciferous vegetables				X	X		X	X	
monoterpenes	citrus fruits					X				
phenolic acids	fruits, vegetables, nuts, tea, coffee	X	X	X	X	X	X	X	X	X
phytic acid	vegetables, cereals							X		
plant sterols	vegetables					X		X		
protease inhibitors	seeds, vegetables, grains						X	X		
selenium	meat(products), eggs, dairy products							X		
vitamin C	(citrus) fruits, vegetables							X		
calcium / vitamin D	dairy products	X								
vitamin E	vegetable oils, whole meal							X	X	
NSAID-like substances (curcumin, resveratrol)	tumeric plant, grape, wine						X	X	X	X

* Mechanisms:
1) Prevention of formation/uptake of carcinogens.
2) Scavenging of (activated) carcinogens.
3) Shielding of nucleophilic sites in DNA.
4) Inhibition of DNA/carcinogen complex.
5) Modifying effect on the activities of xenobiotic-metabolizing enzymes.
6) Modifying effect on the activities of other enzymes.
7) Antioxidant.
8) Induction of apoptosis, reduction of cell growth.
9) Enhancement of intercellular communication
(Blanks do not necessarily indicate lack of effect, but can also indicate lack of information)
(With thanks to Dr Ole Vang, University of Roskilde, Denmark)

Large-scale gene expression techniques in nutrition and colon cancer research

The paragraphs above clearly illustrate the complexity of the process of colon cancer development and diversity of possible mechanisms of protection against cancer development by e.g. food compounds. Large-scale analysis of gene transcripts (mRNA), proteins and metabolites can thus be extremely valuable in this respect. The focus of this thesis is on large-scale gene expression measurement, also known as transcriptomics.

Introduction into transcriptomics

Measurement of gene expression (i.e. mRNA levels) is a valuable tool since many processes are regulated at this level and since it can give a prediction of protein synthesis and for some classes of gene products also of biological activity (e.g. the total enzyme activity towards a substrate).

Initially, mRNA levels were determined using Northern blots or RT-PCR (Reverse Transcription Polymerase Chain Reaction). With this latter technique expression of a gene is compared to expression of a so called 'housekeeping' gene (that has a constant level of expression) by exponential amplification of gene fragments. Recently, this technique was adapted to allow real-time determination (by fluorescent detection) of the amount of target produced after each amplification cycle. The advantage of real-time PCR is the improved method of quantification of gene expression level (without need for gel electrophoresis). cDNA microarrays were developed as a large-scale method for gene expression measurement. Availability of collections (libraries) of gene fragments with a known sequence, often with annotation on their (putative) function in the cell, makes it possible to deposit single-stranded cDNA probes for thousands of different genes in a fixed spot on a surface (e.g. glass slide, plastic, nylon membrane). This collection of thousands of spots on a surface is called a microarray. As in Northern blots, these microarrays make use of the specific hybridization capacity of single stranded DNA and RNA to determine specific mRNA levels of the gene of interest. Before hybridization to the microarray a fluorescent label is incorporated into a cDNA copy of the mRNA, produced by reverse transcription. During subsequent hybridization with the microarray each specific cDNA will only anneal to the complementary cDNA probe for the specific gene that it was derived from. By measurement of fluorescence the amount of labeled cDNA hybridized to each spot can be determined. In practice, two samples are hybridized together on a cDNA microarray, e.g. a test sample and a reference or control sample. In this case, RNA from the test sample is labeled with a fluorophore (e.g. Cy5) and RNA from the reference or control sample is labeled with a different fluorophore (e.g. Cy3). After labeling the derived cDNAs from both samples are pooled and hybridized to the microarray. By measuring fluorescence of both fluorophores a ratio of expression in the test sample compared to the reference or control sample can be calculated. This way, one microarray hybridization can yield expression ratios for ten thousands of genes. Although expression of a large part of these genes will not be different in test and control sample, all

genes that have either a higher or a lower expression in the test sample compared to the control sample can be identified. These differentially expressed genes can provide a wealth of information on cellular mechanisms that were affected in the test sample. This approach can yield information e.g. on effects of treatment of cells with a food compound or on differences between diseased and normal cells. Figure 1.2 gives an overview of the process of labeling and hybridization.

A more detailed overview of strategies and technologies to measure gene expression in cancer research can be found in a review by Liang and Pardee [19].

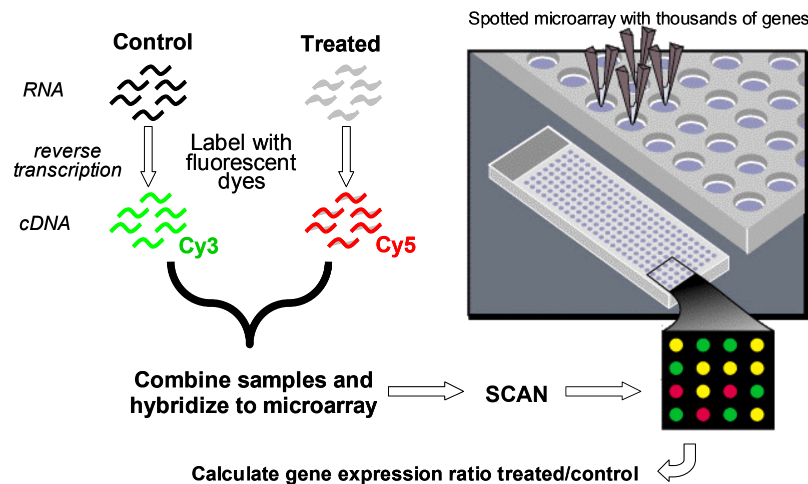

Figure 1.2. Schematic overview of labeling of samples and microarray hybridization

Transcriptomics in colon cancer research

Several studies looked into differences in gene expression between normal colonic tissue and colon tumor tissue using microarrays. One of the first studies in this field was published by Augenlicht and co-workers. A library of 4000 cDNAs derived from HT29 colon cancer cells was spotted and used to measure gene expression profiles in normal human colon tissue and human colon biopsies from different stages in colon carcinogenesis [20]. Since then, several other studies reporting changes in gene expression profiles in different stages of human colon carcinogenesis compared to normal human colon tissue have been published [21-28]. In a few studies colon cells were collected by use of laser-capture microdissection [29, 30]. An overview of these studies is shown in table 1.2. Genes involved in development of metastasis of colon cancer were identified by gene expression profiling of human tissues [31] and by using *in vitro* models [32].

Table 1.2. Overview of studies using gene expression profiling in colon cancer research.

	Description and number of colon biopsies analyzed	Number of genes analyzed and technology used	Number of differentially expressed genes	Remarks
Kitahara *et al.* (2001) [30]	8 tumors + normal colon epithelium	cDNA microarray, 9216 genes	235 genes: 44 up, 191 down	Biopsies collected using laser-capture microdissection
Lin *et al.* (2002) [29]	20 tumors (9 adenomas + 11 adenocarcinomas) + normal colon epithelium	cDNA microarray, 23040 genes	51 up, 376 down	Biopsies collected using laser-capture microdissection
Agrawal *et al.* (2002) [24]	60 tumors (from different disease stages including metastases) + 10 normal mucosa	Oligonucleotide array (Affymetrix), containing 6800 or 12000 elements	>300 (pooled samples); 77 (individual samples) 107 markers for tumor progression	Some biopsies were micro-dissected; analysis of pooled samples (per disease stage) and of individual samples
Notterman *et al.* (2001) [21]	22 tumors (4 adenomas + 18 adenocarcinomas) + normal tissue	Oligonucleotide array (Affymetrix), 6500 and 6800 GeneChip set	19 up, 47 down	
Birkenkamp-Demtroder *et al.* (2002) [23]	Normal tissue (n=6), Dukes stage A (n=5), Dukes stage B (n=6), Dukes stage C (n=6), Dukes stage D (n=4)	Oligonucleotide array (Affymetrix), containing 6500 known genes and 35000 ESTs	157 ESTs 226 known genes: 88 up, 70 down and 68 related to 1 or 2 specific Dukes' stages	RNA was pooled per disease stage group
Frederiksen *et al.* (2003) [27]	Normal tissue (n=5), Dukes stage A (n=5), Dukes stage B (n=5), Dukes stage C (n=5), Dukes stage D (n=5)	Oligonucleotide array (Affymetrix), containing 6800 elements	Between 74 and 200 genes, depending on analysis method	
Zou *et al.* (2002) [25]	9 carcinomas + 8 normal colon	cDNA microarray, 8000 elements	150 up, 100 down	
Takemasa *et al.* (2001) [28]	12 tumors + normal colon tissue	cDNA microarray, 4600 elements	23 up, 36 down	
Williams *et al.* (2003) [26]	20 tumors + normal colon tissue	cDNA microarray, 9592 elements	574 up, 2058 down	
Pinheiro *et al.* (2001) [22]	12 tumors + normal colon tissue	cDNA microarray filter, containing 18376 genes	Many up-regulated genes	Analysis of pool of 9 tumors and pool of 3 tumors
Zhang *et al.* (1997) [33]	2 tumors + normal tissue	SAGE (serial analysis of gene expression)	181 down, 108 up	Also comparison expression in colon cancer cell line with expression in normal tissue

In addition, Zhang et al. used a different technique for expression analysis of many genes (serial analysis of gene expression) to compare expression in human colon cancer tissue with normal colon tissue [33]. Overall, in these large-scale gene expression studies differential expression of tens to hundreds of genes is observed, yielding a substantial amount of colon cancer-specific genes, from which new biomarker genes or biomarker groups can be identified. Also, subsets of genes can be identified that can correctly classify colon tumors of different stages.

When comparing the sets of differentially expressed genes in the studies mentioned in table 2, some genes were repeatedly identified as being colon cancer-related. These include carbonic anhydrase I and II (CA1 and CA2), several keratins and liver fatty acid-binding protein (all lower expressed in colon tumors than in normal tissue) and M-phase inducer phosphatase 2 (CDC25B), transforming growth factor beta induced (TGFBI) and several ribosomal proteins (all higher expressed in colon tumors than in normal tissue).

These genes can be added to the group of colon cancer-related genes that had already been identified and extensively described in other studies, including tumor suppressor genes APC [34, 35] and p53 [36], and oncogenes K-ras [37] and c-myc [38]. Other well-described colon cancer-related genes are cox-2 [39, 40] and mismatch repair genes and other genes related to genetic instability [41]. Altogether, knowledge on genes and proteins related to colon cancer development is growing rapidly by use of large-scale techniques like microarrays.

In addition to above, microarrays were also used to screen human colon cancer cells for hypermethylated genes. Hypermethylation can lead to lower expression of e.g. tumor suppressor genes [42]. Also, *in vitro* studies have identified differentiation-specific markers in colon cancer cells. Genes differentially expressed during differentiation of HT29-D4 cells were identified using differential screening, subtraction hybridization and differential display [43]. Gene expression changes during differentiation of Caco-2 cells were studied using microarrays containing 17280 sequences. During differentiation expression of genes from several functional groups including cell cycle control, DNA synthesis and repair, RNA processing and translation, protein processing and transport, protein degradation, xenobiotic and drug metabolism, kinases and phosphatases and transcription factors was significantly changed [44]. Changes during differentiation of Caco-2 cells were also studied at the protein level, using two-dimensional gel electrophoresis [45].

Food compounds studied using microarray analysis

As described above, many epidemiological studies reported a protective effect of fruits and vegetables on colon cancer development. Many bioactive compounds from plants have been identified. A number of these phytochemicals were studied in this thesis: quercetin and rutin, curcumin, resveratrol, wheat bran and benzyl isothiocyanate. All of these compounds have shown to protect against colon cancer development in rats or in mice [46-52]. Wheat bran is

the grind husk of wheat that consists mainly of dietary fiber. Dietary fiber arrives unchanged in the colon and can be metabolized by colonic microflora into short chain fatty acids. Isothiocyanates are formed after hydrolysis of glucosinolates that are present in cruciferous vegetables. Quercetin is a flavonoid present e.g. in apples and onions that is consumed in relatively large amounts [53]. Flavonoids occur in plants mainly as glycosides. Rutin is a glycoside form of quercetin. Curcumin is a spice and coloring agent derived from the root of the plant *Curcuma longa* that can act as an anti-inflammatory agent and an anti-oxidant [54] [55]. The polyphenol resveratrol is present in skin of grapes and therefore also in wine. It is an anti-oxidant and has anti-inflammatory potential [56, 57].

Although application of expression profiling or microarrays in nutrition research is relatively new, already quite a number of these 'nutrigenomics' publications focusing on colon cancer research can be found. Several studies report effects of short chain fatty acids or specifically butyrate on gene expression in colon cancer cells. Many physiological effects of butyrate in (colon) cancer cells have been reported, both *in vitro* and *in vivo*. These effects include inhibition of cell growth, induction of differentiation and inhibition of histone deacetylase activity [58, 59]. Mariadason et al. [60] studied changes in gene expression in human colon cancer cell line SW620 in response to butyrate at time points between 30 minutes and 48 hours. Gene expression changes indicated a complex reprogramming in SW620 cells induced by butyrate. Effects of butyrate on gene expression was also studied in human colon cancer cell line HT29 [61, 62] and in rat colon cancer cell line CC531 [63]. Several processes or pathways in HT29 cells were affected by butyrate. In addition to genes involved in cell cycle control (including oncogenes and tumor suppressor genes) and apoptosis, these groups of genes included transcription factors, transport-related genes, receptors and extracellular signaling-related genes [64]. In rat colon cancer cell lines butyrate induced changes in expression of about four hundred genes. This study focused on wnt- and ras-signaling target genes [63]. In addition to transcriptome analyses, effect of butyrate on protein expression was also studied in HT29 cells, using 2D gel electrophoresis. Down-regulation of expression of two proteasome subunits by butyrate led to the hypothesis that butyrate might exert its effects partly through altered proteolysis [65].

Other *in vitro* studies analyzed changes in expression profiles of human colon cancer cells (HT29) in response to an aqueous garlic extract [66] or in response to diallyl disulfide, a compound present in garlic [67]. Garlic could play a role in colon cancer prevention. Also, effects of the fatty acid docosahexaenoic acid (DHA) in human colon cancer cells (Caco-2) [68] and role of vitamin D in colon cancer prevention [69] were studied by use of microarrays. Many of the studies looking at mechanisms of food compounds use *in vitro* cultured colon cancer cells. This is an easy to use system for this kind of research. However, it should be kept in mind that effects *in vivo* could be different from the effects found *in vitro*. Also, it is important to consider the concentration range at which a specific effect or mechanism of a

food compound is observed. Some high concentrations tested might never be reached *in vivo*. However, in the intestinal lumen concentrations of food compounds could reach high levels for example after ingestion of supplements. In addition, some compounds are not absorbed in the small intestine, or are completely excreted in the feces, which may result in high concentration of the compounds and their metabolites in the colon.

Only a few *in vivo* studies have used microarrays to study gene expression changes in response to food compounds in gastrointestinal tissue. Expression in scrapings of colon epithelium was measured in rats consuming a diet with PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), a heterocyclic amine, [70] or heme [71]. Both compounds are related to the enhancing effect of red meat on colon cancer incidence. The latter study focused on a novel gene that was strongly down-regulated by heme and could be a biomarker for colonic stress. The effect of the isothiocyanate sulforaphane on gene expression in small intestinal tissue was measured in mice, specifically to study genes regulated by a transcription factor involved in phase-II metabolism [72]. Other studies focused on gene expression changes in liver when studying effects of food compounds. In Min mice (genetic model for intestinal cancer) effect of selenium-enriched broccoli on gene expression was studied in liver, as a model for liver metastasis of colon cancer [73]. Mechanisms of colon cancer prevention by fish oil (rich in n-3 polyunsaturated fatty acids) were studied by comparing gene expression profiles in colonic epithelium of rats fed a high-fat fish oil diet and rats fed a high-fat corn oil diet. Genes involved in oxidative stress response, in cell proliferation, in immune response and in acute phase response were differentially expressed, indicating that these processes could play a role in cancer prevention by fish oil [74]. Also, the effect of the n-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) on gene expression profiles in rat liver was measured, but this study focused on cardiovascular health [75].

Although most attention is given to effects on gastrointestinal tissues, other tissues have also been subjected to microarray studies in the field of nutrition and cancer. Examples are microarray studies of resveratrol effects on ovarian cancer cells [76] and prostate cancer cells [77].

Outline of this thesis

The aim of this thesis is to get more insight in effects and mechanisms of phytochemicals in colon cancer prevention by applying large-scale gene expression measurement techniques. Chapters 2-5 describe the studies carried out with cultured colon (cancer) cells. In chapters 2 and 3 effects of quercetin and curcumin are studied in colon cancer cell lines. In chapter 4 a panel of 14 colon (cancer) cell lines that could be used for screening for effects of food compounds is compared at the gene expression level. In addition, expression profiles of human colon biopsies of normal and tumor tissue are used to generate a subset of colon cancer-specific genes that can be used to relate *in vitro* data to the *in vivo* situation. In chapter 5 two cell lines from the panel were selected to study effects of quercetin, curcumin and resveratrol by gene expression profiling. These plant compounds could possibly protect against development of colon cancer.

In chapter 6 the effect of wheat bran, curcumin, rutin and benzyl isothiocyanate on colon cancer development in rats was studied. In addition, the tumors that developed in these rats were analyzed for gene expression of a number of colon cancer-specific genes. Chapter 7 presents results of gene expression profiling in small intestinal scrapings of the rats in this study. Differences in small intestinal gene expression between the diet groups were related to colon cancer development.

Chapter 8 summarizes the data in this thesis and gives a perspective on the use of transcriptomics (microarrays) and other 'omics' techniques in nutrition research.

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

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Abstract

Introduction. 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 signaling 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 compounds like quercetin.

Introduction

Diet plays an important role both in the development and prevention of colon cancer. Many food compounds 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 comprehensive 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 compounds.

Quercetin is a well-known flavonoid present in plants, consumed in relatively high amounts [4] and with multiple health effects. Quercetin is a potent anti-oxidant 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, that 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 25mM 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 ºC 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'–CTG CAA GGC GAT TAA GTT GGG TAA C-3') and reverse (5'-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-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 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 ($P<0.05$).

Labeling 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 ºC for one hour. After cDNA synthesis, RNA was removed by adding Rnase (15 min, 37 ºC) 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 ºC.
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 ºC, 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 42ºC 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 duplicate 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 2.1), 2) apoptosis (table 2.2), 3) tumor suppressor genes and oncogenes (table 2.3), 4) cell adhesion and cell-cell interaction (table 2.4), 5) transcription (table 2.5), 6) signal transduction (table 2.6) and 7) energy metabolism (table 2.7).

Almost all of the differentially expressed genes involved in cell cycle and differentiation (table 2.1) 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 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 2.2 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 2.1. 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]	-4.06 [a]
AA450062	Prostate differentiation factor	PLAB	-2.42 [*,a]	-2.65 [*,a]
AA074222	Squamous cell carcinoma antigen recognised by T cells	SART1	-2.38 [*,d]	-1.79
T72030	Seven in absentia homolog 1 (Drosophila)	SIAH1	-2.39 [a]	-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	-2.26 [a]	-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	-2.17 [a]	-1.61
AA282936	M-phase phosphoprotein 1	MPHOSPH1	-2.06 [c]	-1.39
AA599145	ZW10 homolog, centromere/kinetochore protein (Drosophila)	ZW10	-2.03	-2.19 [*,c]
AA456291	Developmentally regulated GTP binding protein 2	DRG2	-2.00 [a]	-2.08 [*,c]
R32848	S100 calcium binding protein P	S100P	-1.82 [b]	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	-1.69 [a]	-1.60
AA486208	Cyclin-dependent kinase 4	CDK4	-1.63	-2.17 [b]
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	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 down-regulation, values >0 indicate up-regulation.

[*] 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] : $P<0.001$

Table 2.2. 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 down-regulation, values >0 indicate up-regulation.
[*] 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 2.3. 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 susceptiblity 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 down-regulation, values >0 indicate up-regulation.
[*] 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 2.3 lists tumor suppressor genes and oncogenes. Quercetin upregulated expression of two tumor suppressor 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 2.4). 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, that 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 2.5. 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 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-signaling pathway [43] and can be a target of the p38 MAPK pathway [44]. MITF was upregulated by quercetin.

Table 2.4. Expression changes of genes involved in cell adhesion and cell-cell interaction

			Fold up-/down-regulation	
Accession number	Gene name	Gene symbol	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	-2.20 [a]	-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	-1.40 [c]	1.39
AA190508	A disintegrin and metalloproteinase domain 12 (meltrin alpha)	ADAM12	1.61 [*,c]	1.25
AA676957	Catenin (cadherin-associated protein), α1 (102kD)	CTNNA1	1.63 [b]	1.56 [a]
AA400329	Neurofilament 3 (150kD medium)	NEF3	1.62 [*,c]	1.69 [a]
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	1.92 [a]	1.85 [a]
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	2.08 [a]
H38240	Thrombospondin 2	THBS2	2.25	2.45 [*,a]

These genes were differentially expressed in Caco-2 cells in response to quercetin exposure.
Values <0 indicate down-regulation, values >0 indicate up-regulation.
[*] 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

Table 2.5. 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	-3.42 [a]	-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	-2.20 [a]	-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	-1.87 [a]	-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	-1.78 [b]
AA486533	Early growth response protein 1	EGR1		-2.07 [*,d]
AA099534	Activated RNA polymerase II transcription cofactor 4	PC4	1.59 [a]	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	1.64 [c]	1.47
W96099	Retinoid X receptor-γ	RXRG	1.66	1.78 [*,c]
W47015	Ts translation elongation factor, mitochondrial	TSFM	1.75 [c]	1.79 [b]
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 down-regulation, values >0 indicate up-regulation.
[*] 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

Three members of the MAPK signal transduction pathway were downregulated by quercetin, MAP4K1, MAP3K12 and MAP2K4 (table 2.6). 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 2.7). 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.

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].

Table 2.6. 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 down-regulation, values >0 indicate up-regulation.
[*] 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

Table 2.7. Expression changes of genes involved in energy metabolism

Accession number	Gene name	Gene symbol	Fold up-/down-regulation 5 μM quercetin	Fold up-/down-regulation 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

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 (figure 2.1). 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 (figure 2.1). 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; 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 (figure 2.2). 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 (figure 2.3). 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 (figure 2.3, $P<0.05$ 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.

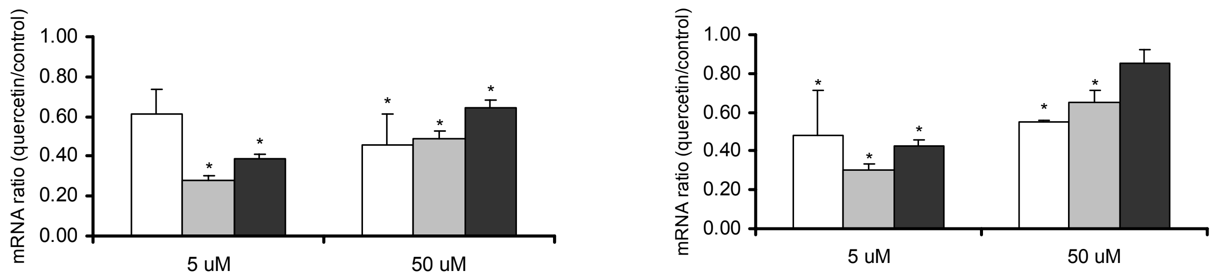

Figure 2.1. CDK4 (left) and RALA (right) gene expression changes in response to quercetin, measured with cDNA microarray (white bars), and measured by real time RT-PCR normalized to beta-actin expression (grey bars) or to GAPDH expression (black bars). Normalized expression is plotted as mRNA ratio (quercetin/control). Values are mean ± standard deviation (n=5). *: expression is significantly different from control (P<0.05)

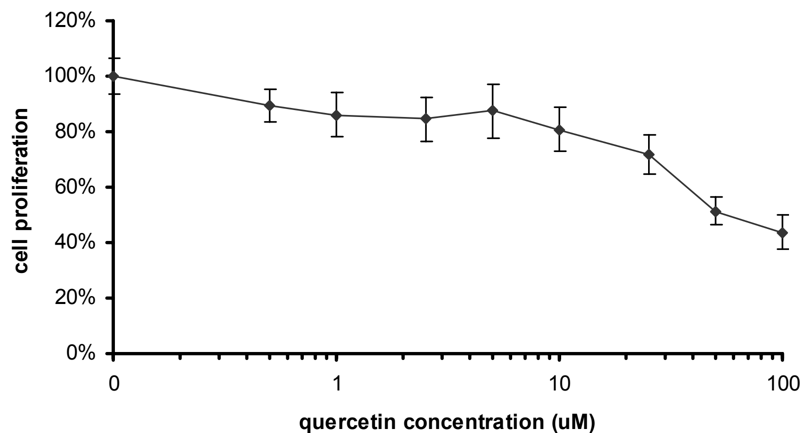

Figure 2.2. Cell proliferation of Caco-2 cells exposed to quercetin for 48 hours, with medium refreshment after 24 hours, measured with BrdU incorporation. Proliferation in cells exposed to solvent control was set to 100%, values are mean ± standard deviation (n=8).

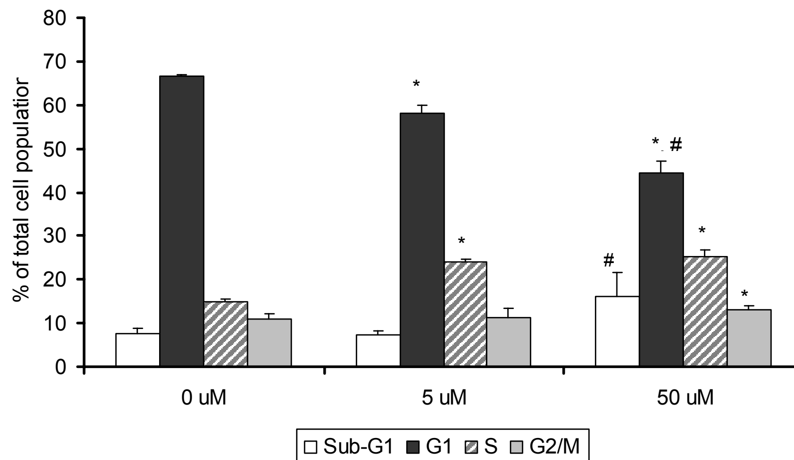

Figure 2.3. Cell cycle distribution of Caco-2 cells exposed to quercetin for 48 hours, with medium refreshment after 24 hours, measured with propidium iodide assay. Values are mean ± standard deviation (n=4). *: significantly different from 0 μM, P<0.05; #: significantly different from 5 μM, P<0.05

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. Additionally, flow cytometric analyses showed a decrease in the percentage of cells 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 2.2, 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 2.3 (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 ß-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 upon 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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Time- and dose-dependent effects of curcumin on gene expression in human colon cancer cells

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Abstract

Introduction. Curcumin is a spice and a coloring food compound with a promising role in colon cancer prevention. Curcumin protects against development of colon tumors in rats treated with a colon carcinogen, in colon cancer cells curcumin can inhibit cell proliferation and induce apoptosis, it is an anti-oxidant and it can act as an anti-inflammatory agent. The aim of this study was to elucidate mechanisms and effect of curcumin in colon cancer cells using gene expression profiling.

Methods. Gene expression changes in response to curcumin exposure were studied in two human colon cancer cell lines, using cDNA microarrays with four thousand human genes. HT29 cells were exposed to two different concentrations of curcumin and gene expression changes were followed in time (3, 6, 12, 24 and 48 hours). Gene expression changes after short-term exposure (3 or 6 hours) to curcumin were also studied in a second cell type, Caco-2 cells.

Results. Gene expression changes (>1.5-fold) were found at all time points. HT29 cells were more sensitive to curcumin than Caco-2 cells. Early response genes were involved in cell cycle, signal transduction, DNA repair, gene transcription, cell adhesion and xenobiotic metabolism. In HT29 cells curcumin modulated a number of cell cycle genes of which several have a role in transition through the G2/M phase. This corresponded to a cell cycle arrest in the G2/M phase as was observed by flow cytometry. Functional groups with a similar expression profile included genes involved in phase-II metabolism that were induced by curcumin after 12 and 24 hours. Expression of some cytochrome P450 genes was downregulated by curcumin in HT29 and Caco-2 cells. In addition, curcumin affected expression of metallothionein genes, tubulin genes, p53 and other genes involved in colon carcinogenesis.

Conclusions. This study has extended knowledge on pathways or processes already reported to be affected by curcumin (cell cycle arrest, phase-II genes). Moreover, potential new leads to genes and pathways that could play a role in colon cancer prevention by curcumin were identified.

Introduction

Curcumin (diferuloylmethane) is a spice and a coloring agent derived from the root of the plant *Curcuma longa* to which colon cancer-preventive properties have been attributed. It is present in curry and mustard, and it is used extensively in Asian countries, also in traditional medicine. The low incidence of colon cancer in Asian countries could be related to low meat intake, but also to the regular use of curcumin in the diet [1].

In rats and mice curcumin has a profound effect on colon carcinogenesis. In rats treated with colon carcinogen azoxymethane (AOM), for example, consuming a diets with 2000 ppm curcumin resulted in a significant reduction of the number of aberrant crypt foci (ACF) after 9 weeks [2] and after 52 weeks incidence and multiplicity of colon adenocarcinomas were significantly reduced [3]. Similarly, incidence and multiplicity of adenomas was decreased in AOM-treated rats fed a diet with 8 or 16 g/kg curcumin for 45 weeks [4]. Also in a mouse model with a mutation in the APC gene curcumin reduced the number of colon tumors [5] or the multiplicity of colon adenomas [6]. Moreover, curcumin was found to protect against development of colon cancer during both the initiation and the promotion stage in AOM-treated rats and AOM-treated mice [7, 8]. In addition to the effect on colon cancer, curcumin showed anticancer effects in intestinal cancer, stomach cancer and hepatocellular carcinoma [9-11].

In vitro, curcumin caused a dose-dependent decrease in cell proliferation in colon cancer cells and the cells accumulated in the G2/M phase [12-14]. Apoptosis was increased in colon cancer cells in response to curcumin [13, 14]. Also in other cell lines, e.g. breast cancer cells, prostate cancer cells and leukemia cells, curcumin inhibited cell proliferation and induced apoptosis [15-19]. In addition to inhibition of cell proliferation and increased apoptosis, many mechanisms have been proposed to explain the anti-carcinogenic effect of curcumin, including its anti-inflammatory and antioxidant activity, induction of phase-II detoxification enzymes, inhibition of cyclooxygenase 2 (COX-2), effect on AP-1 and NFκB transcription factors, inhibition of matrix metalloproteinase (MMP), effect on protein kinases and more [20-22].

In rats absorption of curcumin from the intestine was reported to be about 60% [23]. Curcumin and metabolites formed in intestine and liver are mostly excreted in the feces [24, 25]. As the colon is exposed to both curcumin and its metabolites, it is a likely target for the anticarcinogenic activity of these compounds. Moreover, the fact that humans were able to consume up to 8 grams of curcumin per day without toxic effects [26] makes curcumin a very interesting chemopreventive agent.

New techniques, like multiple gene expression analysis using microarrays, allow for a more comprehensive study of the effects and mechanisms of food compounds. By using cDNA microarrays the expression of thousands of genes can be studied in one experiment. The power of these techniques lies not only in the fact that many genes can be studied in one

experiment, but also in the possibility to identify leads to pathways and mechanisms, as opposed to the extrapolation of assessments of a few genes. In addition, multiple gene expressions can be applied as 'fingerprint' biomarkers.
The aim of our study was to use new and genome-wide information on the gene expression profile induced by curcumin in colon cancer cells to elucidate mechanisms involved in the cancer-preventive action of curcumin. Therefore, HT29 colon cancer cells were exposed to two concentrations of curcumin and gene expression changes were measured at five exposure time points ranging from 3 hours to 48 hours. In addition, the response to short-term exposure to curcumin (3 and 6 hours) was also studied in Caco-2 cells. Furthermore, in HT29 cells changes in expression of cell cycle genes in response to curcumin were related to changes in cell cycle distribution. The time- and concentration-dependent changes in gene expression in HT29 and Caco-2 cells are reported and unique findings and observed similarities are discussed in relation to data from other (microarray) studies to gain more insight into mechanisms of cancer prevention by curcumin.

Methods

Cell culture

HT29 cells (ATCC, Rockville, USA) were grown in McCoy's 5A medium with L-glutamine (Gibco BRL Life Technologies) with 10% FCS. Caco-2 cells (ATCC, Rockville, USA) were grown in DMEM with 25mM HEPES, without sodium pyruvate, with 4500 mg/l glucose, with pyridoxine (Gibco BRL Life Technologies), to which was added 10% FCS, 1% non-essential amino acids (Gibco BRL Life Technologies) and 2% penicillin/streptomycin (Gibco BRL Life Technologies). Cells were maintained at 37 ºC in a humidified atmosphere containing 5% CO_2. Curcumin (Sigma, St. Louis, MO, USA) was dissolved in DMSO. At 70% confluence HT29 cells and Caco-2 cells were exposed to 30 µM (referred to as low concentration) or 100 µM curcumin (referred to as high concentration) for 3 or 6 hours. In addition, HT29 cells were exposed to 25 µM (referred to as low concentration) or 100 µM curcumin (referred to as high concentration) for 12, 24 or 48 hours. The final DMSO concentration in the medium was 0.1%.

Cell cycle analysis using flow cytometry

HT29 cells were plated at a density of $3x10^5$ cells in $25cm^2$ culture flasks. After 24 hours, cells were exposed to curcumin. After the exposure period cells were trypsinized and collected in the original medium (to include floating cells in the analysis). Cells were pelleted by centrifugation at 500xg at 4 ºC and washed with PBS. Cells were resuspended in PBS, ice-cold ethanol (75%) was added to the cells while vortexing and cells were incubated on ice for 1 hour to fixate the cells. Cells were washed with PBS and finally PBS with propidium iodide (Sigma, St. Louis, MO, USA; 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 treated cells and in untreated cells were calculated using a Student's t-test. Also, after trypsinization cells were counted using a Bürker-Turk counting chamber.

RNA isolation

After exposure total RNA was isolated from the cells using Trizol (Life Technologies S.A., Merelbeke, Belgium) according to the manufacturer's protocol. RNA clean-up and Dnase digestion was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA was checked for purity and stability by gel electrophoresis and UV spectrometry. Absorption at 260 and 280 nm was measured and RNA quantity and A_{260}/A_{280} ratio were calculated. Only RNA samples with A_{260}/A_{280} ratio > 1.6 were used in further experiments. Similar to other studies [27] [28], RNA from two or three exposure experiments was pooled before labeling and hybridization to reduce possible bias from single exposure.

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 reactions were performed in a final volume of 25 µl containing 400 nM of each primer, 5 µl of diluted cDNA preparation (equivalent to 50 or 5 ng of starting RNA) 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, AKR1C1 and EGR1 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'; AKR1C1 upstream: 5'- CGT GGG AGG CCG TGG AGA AG-3', downstream: 5'- GCT GCC TGC GGT TGA AGT TGG-3'; EGR1 upstream: 5'- GTC CCC GCT GCA GAT CTC T-3', downstream: 5'- CTC CAG CTT AGG GTA GTT GTC CAT-3'.

Before real-time PCR analyses the specificity of the used primer pairs and 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.

Amplification efficiency for each gene was calculated using a dilution series from a mixture of all cDNAs, as recommended in Pfaffl et al. [29]. A threshold was chosen so that for all

samples in the dilution series amplification was in the exponential phase and the correlation coefficient (as found for the linear relation between threshold cycle value and logarithm of starting quantity) was maximal. This threshold level was also applied to determine the threshold cycle value for all individual samples. Each sample was measured in duplo at two different starting concentrations of cDNA (equivalent to 50 or 5 ng of starting RNA).

cDNA microarray preparation

A set of 4069 sequence-verified human cDNA clones from the I.M.A.G.E. consortium was purchased (Research Genetics, U.S.A.) as PCR products. The cDNA was amplified by PCR with forward (5'–CTG CAA GGC GAT TAA GTT GGGT AAC-3') and reverse (5'-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3') primers. The primers contained a 5'- C6-aminolinker (Isogen Bioscience, Maarsen, The Netherlands) to facilitate cross-linking 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 3x SSC. The clones were spotted 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 in the dark and dust-free until further use.

Labeling and hybridization

Total RNA (25 μg) was labeled using CyScribe first-strand cDNA labeling kit (Amersham Biosciences, Freiburg, Germany). During reverse transcription of the RNA, Cy3- or Cy5-labelled dUTP was built into the cDNA. After incubating the reaction for 1.5 h at 42 ºC, RNA was hydrolyzed by adding NaOH. After neutralization, free nucleotides were removed from the solution using AutoSeq G50 columns (Amersham Biosciences, Freiburg, Germany).

Each sample was labeled twice, once with Cy3 and once with Cy5. cDNA from cells exposed to curcumin (treated sample) was hybridized to the microarray in competition with cDNA from cells exposed to DMSO only (untreated sample), while one of the samples was labeled with Cy3 and the other with Cy5. Hybridizations were repeated with a dye swap.

Before hybridization, Cy3- and Cy5-labeled cDNAs were mixed and human cot-1 DNA (3 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), denatured for 1.5 min at 100 ºC and incubated for 30 minutes at 42 ºC.

Before adding the hybridization mix to the slides, slides were prehybridized in prehybridization buffer (5x SSC, 0.1% SDS and 10 mg/ml bovine serum albumin) for 45 minutes at 42 ºC, 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 42ºC waterbath. After hybridization, slides were washed by submersion and agitation in 0.5x SSC with 0.2% SDS and in 0.5x SSC. Then, slides were firmly shaken in 0.2x SSC and put on a rotation plateau for 10 min. This step was repeated once and slides were dried quickly by centrifugation at 700 rpm.
Slides were scanned with a ScanArray Express confocal laser scanner (Perkin Elmer Life Sciences, USA) and Imagene 4.0 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images, with automatic flagging of weak or negative signals and spots with non-homogeneous signal.

Data analysis

Data were imported into SAS Enterprise guide V2 (SAS Institute Inc., Cary, USA). Spots with a signal/background ratio less than 1.5 or spots that were flagged by the Imagene software were not included in the data analysis. For each spot, local background intensity was subtracted from mean signal intensity and the expression ratio was calculated by dividing background-corrected signal intensity of the treated sample by the background-corrected signal intensity of the untreated sample. Expression ratios were then log transformed (base 2), normalized per slide using an intensity-dependent method (Lowess) [30] and scaled. Data were transferred to Microsoft Excel 97 (Microsoft Corporation, USA). Expression ratios of duplicate (dye swap) arrays were combined and an average expression ratio was calculated, provided that an expression ratio was present for both arrays. In addition, genes that showed specific dye bias at all time point were excluded from further analysis and when looking for early or late response genes only genes that did not show dye bias were included.
Since it is impossible to report results for all genes on the array for all time points, we chose to look at genes with a relatively large change in expression over time. Among these genes we identified early response genes (which were more than 1.5 fold induced or repressed after 3 or 6 hours of exposure) and genes that respond at later time points. In addition, we identified genes with similar responses to curcumin within a pathway and we looked at pathways known to be important in colon carcinogenesis, like cell cycle control and apoptosis.
Clustering methods like K-means clustering (http://ep.ebi.ac.uk/EP/EPCLUST/) were used to identify genes with a similar expression profile over the different time points.

Principal Component Analysis

To analyze and visualize the results of the microarray experiments Principal Component Analysis (PCA) was used, which is a well-known pattern recognition method in the field of multivariate data analysis. Data analysis was performed using the Matlab software (The MathWorks, Inc., 1984-2001) version 6.1.0.450 (R12.1). For PCA analysis the procedure

from the PLS Toolbox was used (Version 2.0.1b 1999, Eigenvector Research, Inc., 1995-1999). Average expression ratios were used in the PCA analysis. Only genes with less than three missing values among the different time points were included. Before PCA analysis, data sets were mean-centered.

Results

Curcumin exposure resulted in gene expression changes in HT29 cells at all time points. However, after exposure to curcumin for 48 hours only 20-30 genes were up- or downregulated more than 1.5 fold, while after for example 3 or 12 hours about 130 genes were up- or downregulated more than 1.5 fold. The total set of gene expression data was submitted to principal component analysis (PCA), identifying the two major components within the total variation between samples, which are then visualized in a two-dimensional plot in which the expression profiles at each of the time points are projected. This PCA plot (Figure 3.1) nicely visualizes the time-dependent changes in the gene expression patterns. Interestingly, the time-dependent shift of the overall gene expression patterns is similar for the low and the high curcumin concentration. The points representing the gene expression patterns after exposure to curcumin for 48 hours are plotted quite distantly from the gene expression patterns at the other time points, also indicating that the expression patterns of the cells exposed to curcumin for 48 hours are different from the gene expression patterns at the other time points.

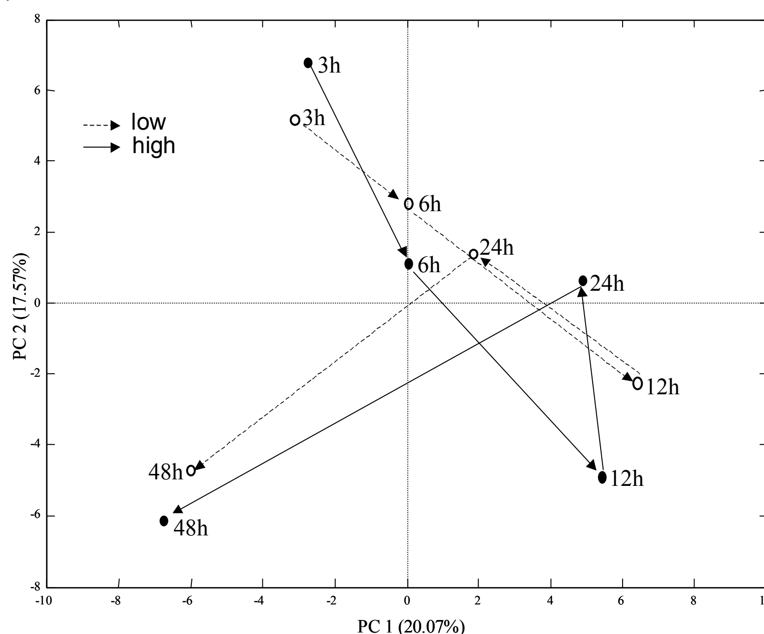

Figure 3.1. Principal Component Analysis (PCA). The gene expression pattern at each time point is visualized as a dot in this two-dimensional graph. The axes show the scores of the gene expression profiles of HT29 cells at the specific time points after exposure to curcumin in principal component (PC) 1 and 2 (which explain the largest part of the variance). The variance explained by the PCs is indicated between parentheses. Open circles and dotted arrows indicate exposure to the low curcumin concentration, filled circles and solid arrows indicate exposure to the high curcumin concentration.

Early response genes were defined as genes that were differentially expressed after exposure to curcumin for 3 or 6 hours. A selection of the early response genes in HT29 cells is listed in table 3.1 (low concentration) and table 3.2 (high concentration). Early response genes were involved in several processes, including cell cycle control, signal transduction, DNA repair, transcription regulation, cell adhesion and xenobiotic metabolism. Both curcumin concentrations caused an increase in expression of genes involved in DNA repair, e.g. MLH1, MSH3 and ERCC2 (Tables 3.1 and 3.2). Upregulated signal transduction genes included STAT3 and STAT5B (Table 3.1) and some genes of the MAPK signal transduction pathway (MAP3K10, MAP4K2; table 3.1 and 3.2). Some other MAPK signal transduction genes were downregulated by the low curcumin concentration (Table 3.1). Expression of a group of genes involved in cell adhesion and protein binding was induced by short-term exposure to curcumin, including annexin (Table 3.1) and integrin genes (Table 3.2). Several genes involved in xenobiotic metabolism were downregulated after short-term exposure to the high concentration of curcumin, namely GSTT2, GSTM4, CYP1B1 (Table 3.2). Expression of GCLC, involved in glutathione synthesis, was upregulated after 6 hours (Table 3.2).

Table 3.1. Early response genes in HT29 cells after exposure to low concentration of curcumin (30 µM).

Accession number	Gene Name	Gene Symbol	Fold up-/down-regulation 3h	6h	Involved in
R10662	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	MLH1	1.90	1.04	DNA repair
AA421716	mutS homolog 3 (E. coli)	MSH3	1.11	1.53	DNA repair
R54492	excision repair cross-complementing rodent repair deficiency, complementation group 2	ERCC2	1.63	1.20	DNA repair
AA399410	signal transducer and activator of transcription 3 (acute-phase response factor)	STAT3	1.56	1.19	Signal transduction/ transcription regulation
AA280647	signal transducer and activator of transcription 5B	STAT5B	1.67	-1.04	Signal transduction/ transcription regulation
AA434420	protein tyrosine phosphatase, non-receptor type 9	PTPN9	1.74	1.64	Signal transduction
H01340	mitogen-activated protein kinase kinase kinase 10	MAP3K10	1.83	1.42	Signal transduction
AA425826	mitogen-activated protein kinase kinase 2	MAP2K2	-1.72	-1.03	Signal transduction
T94169	mitogen-activated protein kinase 8	MAPK8	-1.55	1.16	Signal transduction
R82176	MAD, mothers against decapentaplegic homolog 7	MADH7	-1.77	NA[1]	Signal transduction
N71159	metastasis associated 1	MTA1	1.68	NA	Transcription regulation
AA448256	metal-regulatory transcription factor 1	MTF1	-1.34	-1.66	Transcription regulation
AA465236	forkhead box O3A	FOXO3A	-1.43	-2.01	Transcription regulation
AA775415	SMT3 suppressor of mif two 3	SMT3H2	-1.77	-1.57	Nuclear transport

Accession number	Gene Name	Gene Symbol	Fold up-/down-regulation 3h	6h	Involved in
	homolog 2 (yeast)				
T54121	cyclin E1	CCNE1	1.63	NA	Cell cycle
N74285	CDC5 cell division cycle 5-like	CDC5L	1.51	1.05	Cell cycle
AA789328	cyclin-dependent kinase (CDC2-like) 10	CDK10	1.51	NA	Cell cycle
AA877595	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	CDKN2A	1.49	1.86	Cell cycle
AA488324	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	BUB1B	-1.95	NA	Cell cycle
R19158	serine/threonine kinase 6	STK6	-1.25	-1.80	Cell cycle
AA071486	serine/threonine kinase 12	STK12	-1.50	-1.83	Cell cycle
AA082943	cyclin G1	CCNG1	-1.54	1.24	Cell cycle
W51794	matrix metalloproteinase 3 (stromelysin 1, progelatinase)	MMP3	-1.12	1.52	Breakdown of extracellular matrix
N33214	matrix metalloproteinase 14 (membrane-inserted)	MMP14	-1.53	NA	Breakdown of extracellular matrix
AA406571	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	CEACAM1	1.76	1.18	Cell adhesion
AA464982	annexin A11	ANXA11	1.75	1.13	Protein binding
AA419015	annexin A4	ANXA4	NA	1.79	Protein binding
AA856874	furin (paired basic amino acid cleaving enzyme)	FURIN	1.87	1.48	Proprotein convertase
R26186	protein phosphatase 1, catalytic subunit, beta isoform	PPP1CB	1.64	1.65	Phosphatase activity
N28497	protein phosphatase 2 (formerly 2A), regulatory subunit A (PR 65), beta isoform	PPP2R1B	1.91	NA	Phosphatase activity
AA877845	LIM domain kinase 2	LIMK2	1.88	1.56	Protein-protein interactions
AA634028	major histocompatibility complex, class II, DP alpha 1	HLA-DPA1	-2.06	NA	Immune-related
T63324	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1	1.60	1.17	Immune-related
H95960	secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	-1.13	-1.79	Collagen-binding Calcium-binding
AA456291	Developmentally regulated GTP binding protein 2	DRG2	1.67	1.47	GTP-binding protein
AA430178	RAN binding protein 2-like 1	RANBP2L1	-1.85	-1.11	GTP-binding protein

Values <0 indicate down-regulation, values > 0 indicate up-regulation.
[1] NA: not available

Table 3.2. Early response genes in HT29 cells after exposure to high concentration of curcumin (100 μM).

Accession number	Gene Name	Gene Symbol	Fold up-/down-regulation 3h	6h	Involved in
AA421716	mutS homolog 3 (E. coli)	MSH3	1.52	1.47	DNA repair
R91570	signal transducer and activator of transcription 4	STAT4	1.17	-1.57	Signal transduction
H01340	mitogen-activated protein kinase kinase kinase 10	MAP3K10	1.81	1.33	Signal transduction
R50953	mitogen-activated protein kinase kinase kinase kinase 2	MAP4K2	1.65	NA[1]	Signal transduction
R38343	protein tyrosine phosphatase, receptor type, G	PTPRG	1.56	1.05	Signal transduction
AA486533	early growth response 1	EGR1	8.66	1.29	Transcription regulation
AA600217	activating transcription factor 4 (tax-responsive enhancer element B67)	ATF4	1.94	NA	Transcription regulation
R83270	TGFB-induced factor (TALE family homeobox)	TGIF	-1.97	-1.06	Transcription regulation
AA733038	polymerase (RNA) I polypeptide C, 30kDa	POLR1C	-1.66	-1.09	Transcription regulation
AA857163	amphiregulin (schwannoma-derived growth factor)	AREG	1.98	1.23	Growth factor (cell cycle)
R45059	vascular endothelial growth factor	VEGF	1.78	NA	Growth factor (cell cycle)
R54846	fibroblast growth factor receptor 1	FGFR1	1.78	NA	Cell growth (signal transduction)
AA877595	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	CDKN2A	1.72	NA	Cell cycle
AA450265	proliferating cell nuclear antigen	PCNA	-1.59	-1.09	Cell cycle
N74956	insulin-like growth factor binding protein 7	IGFBP7	-1.55	-1.39	Cell proliferation
AA180742	tubulin, alpha 1 (testis specific)	TUBA1	-1.53	-1.09	Cytoskeleton
AA480995	methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	MTHFD2	1.79	1.56	Single carbon unit transfer
H23187	carbonic anhydrase II	CA2	-1.09	-1.69	Single carbon unit transfer
AA037229	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	ITGB3	-1.21	-1.50	Cell adhesion
AA485668	Integrin, beta 4	ITGB4	1.82	1.21	Cell adhesion
W56754	Integrin, beta 8	ITGB8	1.51	-1.08	Cell adhesion
R48796	integrin, alpha L (antigen CD11A (p180), lymphocyte function-associated antigen	ITGAL	1.50	1.19	Cell adhesion
AA159577	mucin 5, subtype B, tracheobronchial	MUC5B	1.84	1.27	Extracellular matrix formation
AA490208	glutathione S-transferase theta 2	GSTT2	-1.56	-1.48	Xenobiotic metabolism
AA486570	glutathione S-transferase M4	GSTM4	-1.13	-1.54	Xenobiotic metabolism
AA448157	cytochrome P450, family 1, subfamily B, polypeptide 1	CYP1B1	-1.74	-1.33	Xenobiotic metabolism
H56069	glutamate-cysteine ligase, catalytic subunit	GCLC	-1.16	1.52	Glutathione synthesis
AA759046	dual specificity phosphatase 2	DUSP2	2.31	1.24	Inactivation MAPK family kinases

Accession number	Gene Name	Gene Symbol	Fold up-/down-regulation 3h	6h	Involved in
AA187349	ferredoxin 1	FDX1	-1.57	NA	Electron transfer, metabolism
AA450205	translocation protein 1	TLOC1	-1.54	-1.54	Protein transport
H13691	major histocompatibility complex, class II, DM beta	HLA-DMB	1.51	-1.01	Immune-related
T70057	immunoglobulin J polypeptide	IGJ	-1.91	1.02	Immune-related
AA496359	immediate early protein	ETR101	1.54	1.10	Unknown
AA425687	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1	DDX1	-1.56	NA	Unknown

Values <0 indicate down-regulation, values > 0 indicate up-regulation.
[1] NA: not available

Also, genes involved in transcription regulation were induced or repressed by short-term exposure to curcumin. Among these were transcription factors such as activating transcription factor 4 (ATF4) and early growth response 1 (EGR1) (Table 3.2). One of the target genes regulated by ATF4 is asparagine synthetase (ASNS) [31]. Figure 3.2 shows the expression profile of ATF4 and ASNS in response to curcumin. Induction of ATF4 expression at the early time points is followed by induction of expression of ASNS at the same and later time points. EGR1, a transcription factor involved in cell growth regulation and tumor suppression [32], was the most upregulated early response gene. In contrast to this strong upregulation after exposure to the high concentration of curcumin, this gene was not induced by the low curcumin concentration (Figure 3.3). Expression of EGR1 was also measured by real-time RT-PCR. The induction factor for EGR1 after 3 hours of exposure to the high curcumin concentration was even higher when measured with real-time PCR than with the cDNA microarray (23.0 ± 2.73 when normalized to beta-actin and 30.5 ± 2.96 when normalized to GAPDH versus 8.7 as measured on cDNA microarray). Similarly, downregulation of EGR1 after 12 hours (2-fold) and upregulation after 24 hours of exposure (1.4-fold) were confirmed by real-time RT-PCR analysis of the effects on EGR1 expression (data not shown).

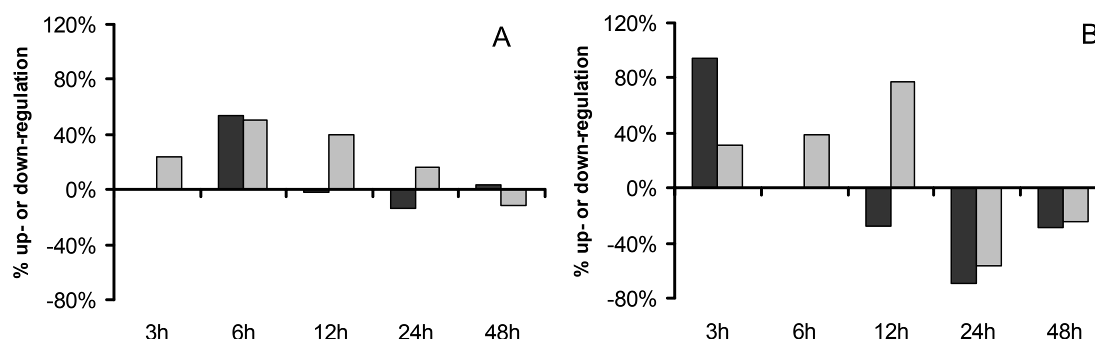

Figure 3.2. Expression profile of activating transcription factor 4 (ATF4, black bars) and asparagine synthetase (ASNS, grey bars) in response to exposure to curcumin. A: low concentration, B: high concentration.

In addition to the functional groups mentioned above several genes involved in the cell cycle or cell growth were among the early response genes. For example several growth factors (AREG, VEGF, FGFR1) were upregulated three hours after exposure to the high concentration of curcumin (Table 3.2). At the same time point, expression of cyclin-dependent kinase inhibitor p16INK4 (CDKN2A) was upregulated (Table 3.2). Expression of cell growth related genes PCNA and IGFBP7 was downregulated by the high concentration of curcumin (Table 3.2). Also after exposure to the low curcumin concentration some cell cycle genes were downregulated, like BUB1B (a mitotic checkpoint gene), STK6, STK12 and cyclin G1 (CCNG1). Upregulated cell cycle genes included CDK10, CDC5L and cyclin E1 (CCNE1), but also cell cycle inhibitor p16INK4 (CDKN2A) (Table 3.1). Also at later time points differential expression of genes involved in cell cycle or cell growth was found. One of the most strongly downregulated genes in HT29 cells was polo-like kinase (PLK), after exposure to the high curcumin concentration for 24 hours (figure 3.3). PLK is a cell cycle gene involved in spindle assembly. It is expressed at a higher level in colorectal cancer than in normal colon tissue [33]. Downregulation of PLK has been shown to inhibit cell growth in cancer cells [34].

Several histone genes (H3F3A, HIST1H4C) were downregulated by curcumin, especially at the 12h and 24h time points (data not shown). Histone deacetylase (HDAC1) was downregulated 1.7-fold by the high concentration of curcumin after 24 hours. Other genes involved in cell cycle control that were differentially expressed after 12 or 24 hour exposure to high concentration curcumin were retinoblastoma 1 (RB1), MAD2L1, BUB1, cyclin G1 (downregulated 1.6 to 2-fold). In response to exposure to the low concentration of curcumin for 12 hours expression of cyclin H was downregulated (1.5-fold) and expression of cyclin A2 was upregulated (1.7-fold). In contrast to cell cycle-related genes, only a few genes involved in apoptosis were differentially expressed in response to curcumin. The most striking effect was observed with programmed cell death 2 (PDCD2), which was downregulated 1.8-fold by the high concentration of curcumin after exposure for 12 hours.

When looking in detail into the gene expression profiles at the different time-points, several genes known to be involved in colon carcinogenesis were found that responded to curcumin exposure. Expression changes of these genes are shown in figure 3.3. Protein expression of urokinase-type plasminogen activator (PLAU) was reported to be higher in colon tumours than in normal colon tissue [35]. In our study, curcumin downregulated expression of PLAU in HT29 cells (Figure 3.3). In addition, expression of urokinase-type plasminogen activator receptor (PLAUR) was upregulated by curcumin (Figure 3.3). Recently, it was shown that members of the transmembrane 4 superfamily could play a role in colon cancer [36]. Two members of this family, TM4SF1 and TM4SF4, showed differential responses to exposure to curcumin (Figure 3.3). Expression of carbonic anhydrase 2 (CA2) was found to be downregulated in colon tumors [37, 38]. In this study, expression of CA2 was downregulated

in response to exposure to curcumin (Figure 3.3). Expression of protein kinase B (AKT1) was upregulated by the low curcumin concentration after 3 hours and by the high curcumin concentration after 24 hours (Figure 3.3). Protein kinase B/Akt is an important part of signal transduction pathways regulating for example apoptosis. Curcumin downregulated expression of tumor protein p53 (TP53) (Figure 3.3), a transcription factor that has a role in cell cycle control [39]. Expression of methylene tetrahydrofolate dehydrogenase/cyclohydrolase (MTHFD2) was upregulated at early time points and downregulated at the later time points. This gene has a role in the maintenance of the single carbon metabolic pool carried by folate. Folate could be involved in colorectal carcinogenesis through DNA methylation and polymorphisms in genes involved in folate metabolism [40].

Clustering methods were used to identify groups of genes with similar expression profile across the different time points. Several functional groups of genes with a similar expression profile were found. Figure 3.4 shows the response of the cluster of genes involved in phase-II metabolism (biotransformation of electrophilic mutagens and other toxic compounds) to exposure to curcumin at the different time points. Curcumin induced expression of these genes, with a maximum induction after exposure for 12 or 24 hours. After 48 hours no differential expression is seen. The most upregulated gene is aldo-keto reductase family 1 member C1 (AKR1C1). The expression changes of AKR1C1 in response to curcumin were confirmed with real-time RT-PCR (Figure 3.5). This gene, also known as dihydrodiol dehydrogenase, was recently shown to be underexpressed in 50% of gastric cancers [41]. Previously it was also shown that gene expression of AKR1C1 could be upregulated in HT29 cells by phenolic antioxidants [42]. Isothiocyanates induced protein expression of AKR1C1, NAD(P)H:quinone oxidoreductase 1 (NQO1) and heavy subunit of glutamate-cysteine ligase (GCLC) in a colon cancer cell line [43]. These genes were also upregulated by curcumin in this study. In addition, NQO2 and epoxide hydrolase 1 (EPHX1) were also upregulated (Figure 3.4). Several other redox-sensitive genes were upregulated after short-term exposure to the high concentration of curcumin, like AREG, ATF4, EGR1, FGFR1 (Table 3.2) [44].

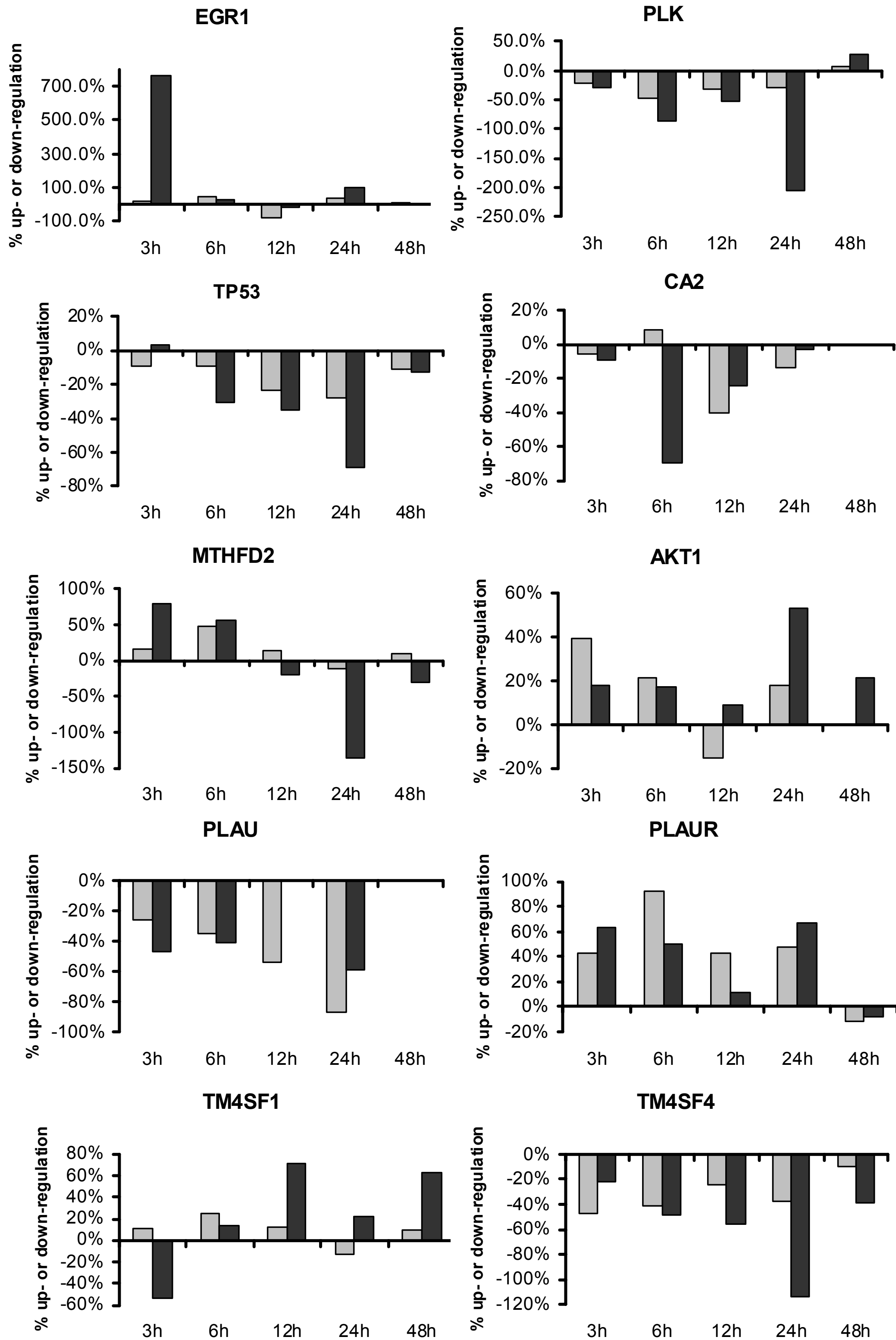

Figure 3.3. Expression profile of genes in response to curcumin; grey bars indicated the low concentration and black bars indicate the high concentration. EGR1: early growth response 1; PLK: polo-like kinase; TP53: tumor protein p53; CA2: carbonic anhydrase 2; MTHFD2: methylene tetrahydrofolate dehydrogenase/cyclohydrolase; AKT1: protein kinase B/Akt; PLAU: urokinase-type plasminogen activator; PLAUR: urokinase-type plasminogen activator receptor; TM4SF1: transmembrane 4 superfamily member 1; TM4SF4: transmembrane 4 superfamily member 4.

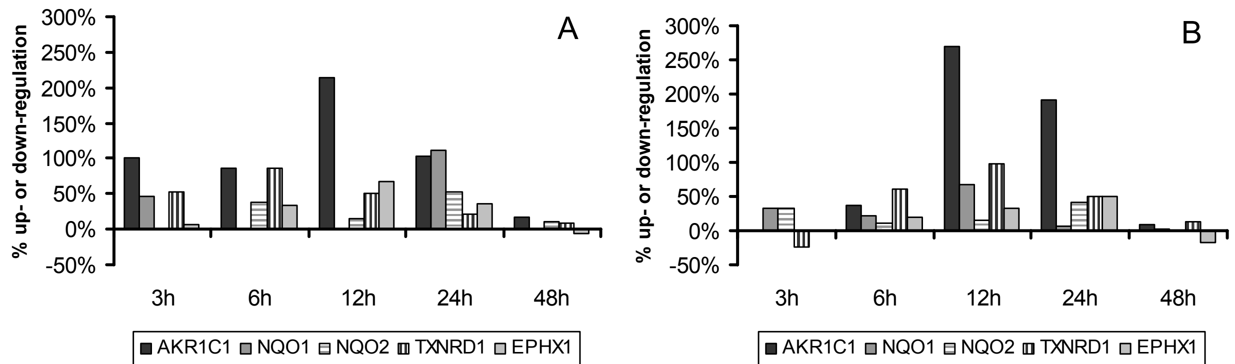

Figure 3.4. Response of genes involved in phase II metabolism to exposure to curcumin; A: low concentration, B: high concentration. AKR1C1: aldo-keto reductase family 1 member C1; NQO1: NAD(P)H dehydrogenase, quinone 1; NQO2: NAD(P)H dehydrogenase, quinone 2; TXNRD1: thioredoxin reductase 1; EPHX1: epoxide hydrolase 1.

However, not all phase-II genes were upregulated by curcumin. In this study several GSTs were downregulated, e.g. GSTT2 and GSTM4 (table 3.2), GSTZ1 (after exposure for 24 hours). In addition, GCLM (the light regulatory subunit of gamma-glutamylcysteine synthetase) was downregulated by curcumin with maximal downregulation by the low concentration of curcumin after 12 hours (1.8-fold) and by the high concentration of curcumin after 24 hours (1.9-fold). Gamma-glutamyl hydrolase (GGH) was downregulated 1.5-fold by the high curcumin concentration after 24 hours. Several phase-I metabolism genes were downregulated by curcumin, for example expression of CYP1B1 was inhibited by the high curcumin concentration after 3 hours (Table 3.2) and expression of some other cytochrome P450 genes was downregulated after exposure for 12 hours to the high curcumin concentration (data not shown). Expression of the aryl hydrocarbon receptor (AHR) was also slightly downregulated at this point (1.4-fold).

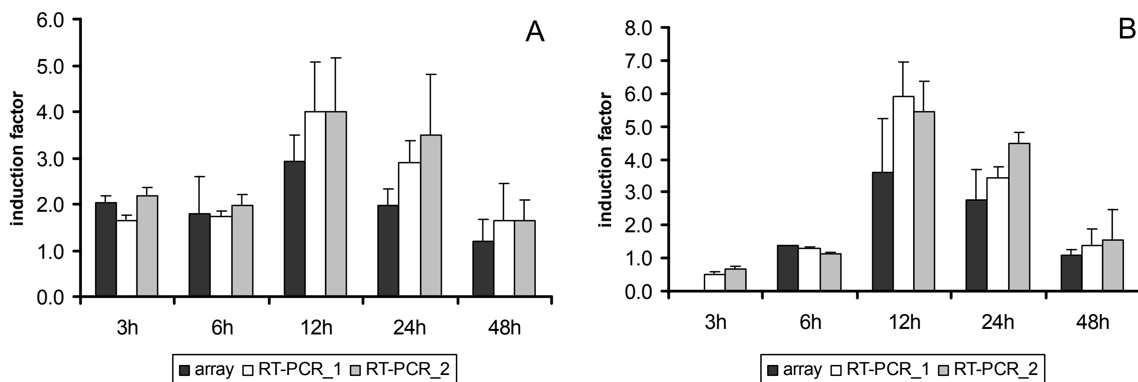

Figure 3.5. AKR1C1 gene expression changes in response to curcumin [A: low concentration, B: high concentration], measured with cDNA microarray (black bars), and measured by real time RT-PCR normalized to beta-actin expression (white bars) or to GAPDH expression (grey bars). Expression is plotted as induction factors (level in treated cells compared to untreated cells) (mean ± standard deviation).

A second group of genes that showed a similar response to curcumin exposure in this study were metallothionein genes. All were downregulated by curcumin, specifically at the 12 and 24 hour time points (Figure 3.6). In addition, the metallothionein downregulation was preceded by a downregulation of MTF1, the transcription factor regulating metallothionein expression, by the low curcumin concentration (Table 3.1). In human colon cancer cells metallothionein was found to be a possible proliferation marker, as it is higher expressed in proliferating cells and its expression is cell cycle regulated [45]. Expression of metallothionein in colon tumors is associated with poor prognosis [46].
Another group of genes that responded to curcumin exposure in a similar fashion were tubulin genes. These genes were downregulated after 3 hours, but were upregulated after 48 hours of exposure to the high curcumin concentration (Figure 3.7). Alpha tubulin has been reported to be differentially expressed in normal colon compared to colon tumors [47] and alpha tubulin was downregulated during differentiation of HT29-D4 cells [48].
Several proteasome genes were upregulated by the high concentration of curcumin after 12 or 24 hours: PSMA1, PSMA7, PSMB2 after 12 hours and PSMB6, PSMC4, PSMD2 after 24 hours (data not shown).

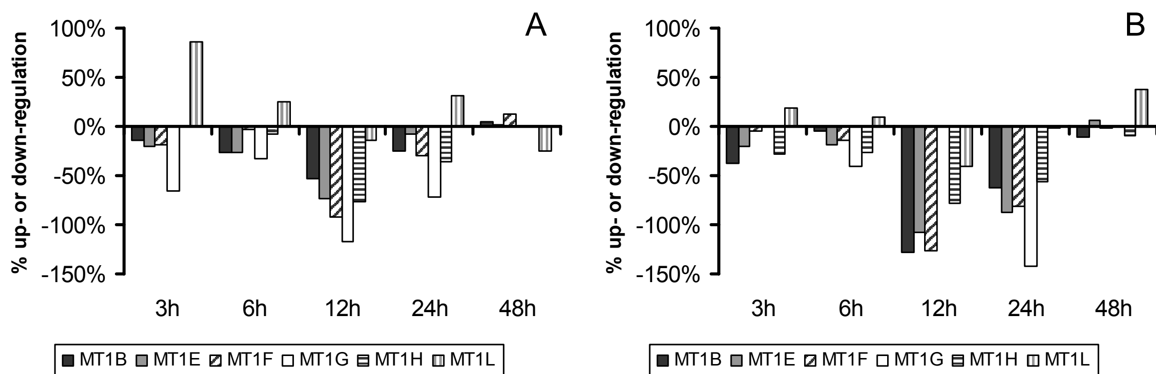

Figure 3.6. Response of metallothionein (MT) genes 1B-1L to exposure to curcumin; A: low concentration, B: high concentration.

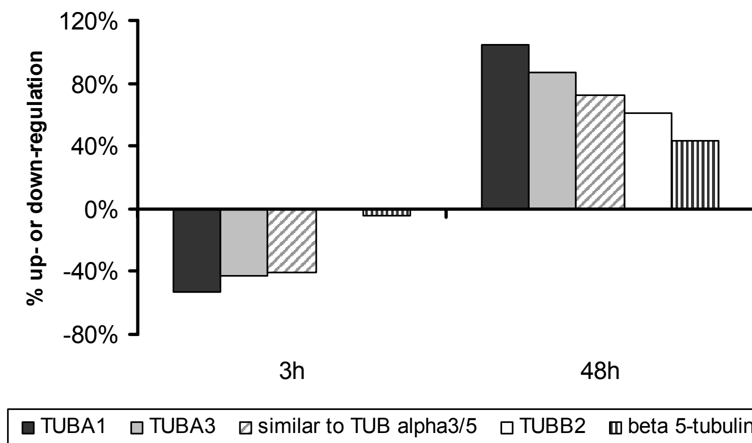

Figure 3.7. Response of tubulin genes in HT29 cells to exposure to curcumin (high concentration); TUBA1: tubulin, alpha 1; TUBA3: tubulin, alpha 3; TUBB2: tubulin, beta 2.

In addition to HT29 cells, expression changes at early time points of exposure to curcumin were also studied in Caco-2 cells. Fewer genes were differentially expressed in these cells than in HT29 cells, between 20 and 50 genes were up- or downregulated more than 1.5 fold after short-term exposure to curcumin. The most striking response in Caco-2 cells was a strong downregulation of CYP1A1 expression after exposure to both concentrations of curcumin for 3 and 6 hours (Table 3.3). CYP1A1 expression did not change in HT29 cells in response to curcumin, however CYP1B1 expression was downregulated by the high concentration of curcumin (Table 3.2). Some genes showed a similar response in Caco-2 cells as in HT29 cells, for example EGR1 and AKR1C1. However, upregulation in Caco-2 cells (Table 3.3) was not as strong as in HT29 cells (Figure 3.4 and 3.3). Expression of TLOC1 and POLR1C was downregulated in Caco-2 cells (Table 3.3) as well as in HT29 cells after short-term exposure (Table 3.1 and 3.2). Also upregulation of ETR101 and an integrin gene in Caco-2 cells is consistent with the results in HT29 cells. In Caco-2 cells several transcription factors were upregulated by curcumin, like NR2F1, NR4A1 and MTF1, whereas transcription factor FOSB and transcription-related genes HNRPA0 and POLR1C were downregulated (Table 3.3).

Table 3.3. Early response genes in Caco-2 cells after exposure to curcumin.

			Fold up-/down-regulation				
			30 µM		100 µM		
Accession number	Gene Name	Gene Symbol	3h	6h	3h	6h	Involved in
AA418907	cytochrome P450, family 1, subfamily A, polypeptide 1	CYP1A1	-2.93	-3.00	-2.89	-6.39	Xenobiotic metabolism
R93124	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1)	AKR1C1	1.05	1.60	-1.15	1.18	Xenobiotic metabolism
AA486533	early growth response 1	EGR1	1.11	1.03	2.49	2.30	Gene transcription
AA452909	nuclear receptor subfamily 2, group F, member 1	NR2F1	-1.40	NA[1]	-1.20	1.83	Gene transcription
N94487	nuclear receptor subfamily 4, group A, member 1	NR4A1	-1.19	1.09	1.45	1.08	Gene transcription
AA448256	metal-regulatory transcription factor 1	MTF1	0.28	0.34	0.09	0.74	Gene transcription
T61948	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	-1.37	1.21	-1.22	-1.62	Gene transcription
AA599176	heterogeneous nuclear ribonucleoprotein A0	HNRPA0	-1.05	-1.31	-1.12	-1.58	Gene transcription
AA733038	polymerase (RNA) I polypeptide C, 30kDa	POLR1C	-1.22	-1.28	-1.34	-1.73	Gene transcription
AA464970	phospholipase C, beta 2	PLCB2	NA	1.93	1.08	1.05	Signal transduction
AA450205	translocation protein 1	TLOC1	1.15	-1.36	-1.20	-1.62	Protein transport
AA419177	solute carrier family 7, member 5	SLC7A5	-1.27	-1.54	-1.56	-1.99	Transport
AA425451	integrin, alpha E	ITGAE	NA	1.43	1.54	NA	Cell adhesion
AA888148	tubulin, beta 2	TUBB2	-1.25	-1.25	-1.57	-1.97	Cytoskeleton
AA496359	immediate early protein	ETR101	-1.09	-1.02	1.77	1.52	unknown

Values <0 indicate down-regulation, values > 0 indicate up-regulation.
[1] NA: not available

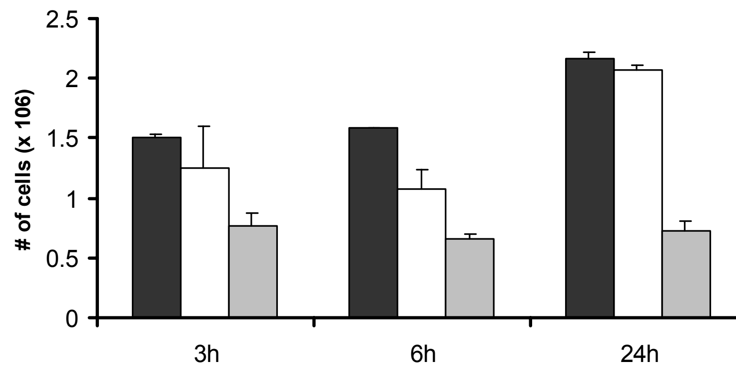

Figure 3.8. Number of HT29 cells after exposure to curcumin at different time points. Black bars: untreated cells, white bars: low concentration, grey bars: high concentration. Values are mean ± standard deviation.

When HT29 cells were exposed to the low curcumin concentration for 6 hours, the number of cells was decreased compared to cells not exposed to curcumin (Figure 3.8). However, after 24 hours a difference with cells that were not exposed to curcumin was no longer seen. Exposure of the cells to the high concentration of curcumin caused a decrease in cell number at all time points (Figure 3.8). After exposure to curcumin for 3 or 6 hours a significant decrease in the percentage of cells in the G1 phase of the cell cycle and a significant increase in the percentage of cells in the G2/M phase of the cell cycle was observed (Figure 3.9). This effect was dose-dependent. At the 24 hour time point the percentage of cells in the G1 phase was decreased significantly and the percentages of cells in both the S and the G2/M phase were increased significantly (Figure 3.9). When absolute cell numbers were recalculated from the percentages, the number of cells in G1 and S phase decreased significantly after exposure for 3 hours (only for the high curcumin concentration) or 6 hours. The absolute number of cells in G2/M phase remained constant. After exposure to the low concentration of curcumin for 24 hours, the absolute number of cells in G1 phase decreased significantly, but the absolute number of cells in S and G2/M phase increased significantly (data not shown).

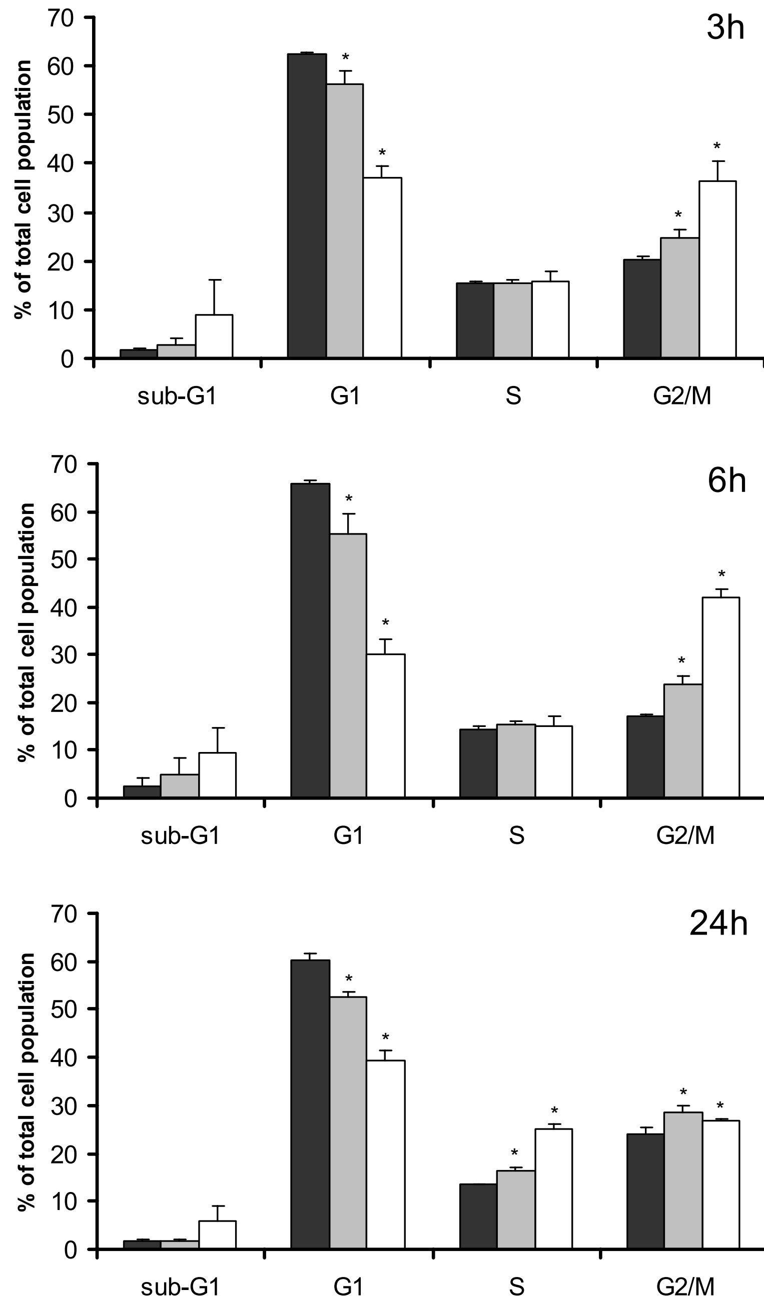

Figure 3.9. Cell cycle distribution of HT29 cells exposed to curcumin for 3, 6 or 24 hours, measured with propidium iodide assay. Black bars: untreated cells, grey bars: low concentration, white bars: high concentration. Values are mean ± standard deviation (n=3 or 4). *: significantly different from untreated cells, P<0.05

Discussion

In this study the effect of curcumin on gene expression in HT29 colon cancer cells is determined at different time points. In addition, gene expression changes in response to short term exposure to curcumin were also studied in Caco-2 cells. The concentrations of curcumin (in the micromolar range) used in this study reflect the *in vivo* situation. Based on almost complete fecal excretion of curcumin and its metabolites [24, 25] and assuming a volume of one liter in the stomach and a 10-fold dilution from stomach to colon, the concentration of curcumin in the intestinal lumen can be as high as 270 μM after consuming one gram of curcumin, with a meal and/or as supplements. A similar concentration range for curcumin in colon was reported by Wortelboer et al. [49]. The highest concentration used in this study was 100 μM. Exposure of HT29 cells to this curcumin concentration resulted in a decrease in cell number and floating cells were seen in the culture flasks after 24 or 48 hours. Therefore, it is possible that gene expression changes after exposure to 100 μM curcumin for longer time periods (24, 48 hours) were related to toxic effects of the compound.

The study set-up with a time series of exposure to curcumin allowed us to identify early response genes and gene expression changes over time. Early response genes were identified; these were involved in DNA repair, signal transduction, transcription regulation, cell adhesion, xenobiotic metabolism. Also, genes involved in cell cycle control were up- or downregulated by short-term exposure to curcumin. Additionally, we have shown that exposure of HT29 cells to curcumin for 3 or 6 hours results in a decrease in percentage of cells in G1 phase and an increase in percentage of cells in G2/M phase. Other studies also reported a cell cycle arrest in the G2/M phase in colon cancer cells after curcumin exposure (similar concentrations, in micromolar range), but only exposure periods of 12 hours or longer were studied [12-14]. In addition, after 24 hours an increase in the percentage of cells in the S phase was observed. Hanif et al. [12] also found an increase in the percentage of cells in the S-phase in HT29 cells after exposure to curcumin for 24 hours. Interestingly, the initial decrease in cell number after short-term exposure to the low concentration of curcumin was no longer seen after 24 hours. At this time point, exposure to the low curcumin concentration had resulted in a significant increase in the absolute number of cells in the S and G2/M phase when compared to cells that were not exposed to curcumin. This indicates that the cells may have overcome the cell growth-inhibiting effect of curcumin at this time point. Consistent with this cell-physiological observation, changes in expression of cell cycle genes were not found after exposure to the low curcumin concentration for 24 hours.

The downregulation of expression of PLK by curcumin as found in this study could be involved in the G2/M arrest. Recently it was shown that downregulation of PLK in colon cancer cell line SW480 resulted in an increase in the percentage of cells in the G2/M phase [50]. Also in HeLa cells, depletion of PLK resulted in G2/M phase arrest and apoptosis [51]. Also other genes involved in transition through the G2/M phase of the cell cycle were

downregulated by curcumin, like BUB1B and MAD2L1. Downregulation of HDAC1 by curcumin could possibly also be related to the S/G2/M phase arrest, since exposure of leukaemia cells to histone deacetylase inhibitors resulted in a G2/M phase arrest [52]. In cancer cells inhibition of histone deacetylase activity can cause growth arrest and apoptosis, and thus inhibit carcinogenesis [53]. Recently, it was reported that HDAC1 is associated with PCNA [54]. PCNA, involved in DNA replication, was also downregulated by curcumin.

It is known that dietary curcumin can induce activity of antioxidant or phase-II enzymes in livers of rats treated with 1,2-dimethylhydrazine (DMH) or in liver and kidney of ddY mice [55, 56]. In this study, it was found that curcumin also has an effect on phase-II enzyme gene expression level in colonic cells. Induction of phase-II genes can be a mechanism to protect against development of cancer [57]. Expression of most of these genes is induced through the antioxidant response element (also known as EpRE) [58] [59]. In human bronchial epithelial cells curcumin increased EpRE-binding activity and changed the composition of the EpRE-binding complex [60]. One of the transcription factors present in the EpRE-binding complex is Nrf2. Curcumin exposure resulted in an increase in nuclear Nrf2 content in bronchial epithelial cells [60] and in increased expression of Nrf2 in renal epithelial cells [61]. Unfortunately, Nrf2 was not represented on the cDNA microarray used in this study. Possibly, transcription factor ATF4 can form a dimer with Nrf2 [62], suggesting a role in EpRE-mediated effects. Indeed, curcumin was found to upregulate expression of ATF4 at the early time points, consistent with its role as an initiator of the curcumin effect through gene transcription modulation. Curcumin downregulated expression of several GST genes. This corresponds to an earlier observation that GSTP1 expression was downregulated by curcumin in leukemia cells [63]. Overall, the effect of curcumin on the phase-II and redox-sensitive genes could indicate an increased level of protection of the cells against oxidative stress, consistent with its function as an anti-oxidant. In addition to its effect on phase-II biotransformation genes, it is suggested that curcumin can inhibit activation of carcinogens by cytochrome P450 enzymes [64]. In Caco-2 cells curcumin caused a strong downregulation of CYP1A1 gene expression. In HT29 cells curcumin downregulated expression of the CYP1B1 and aryl hydrocarbon receptor genes.

Changes in expression of cell cycle-related genes together with induced cell cycle arrest as well as induction of phase-II genes can be mechanisms of colon cancer prevention. Differential expression of tubulin genes and of proteasome genes in response to curcumin could also be interesting in view of the anticarcinogenic effect of curcumin. Microtubules formed by tubulin are important for spindle formation during cell division [65]. Protein degradation by proteasomes plays a role in cell cycle control and apoptosis and proteasome inhibitors are tested for use in anticancer therapy [66]. Furthermore, downregulation of p53 expression could also play a role. Rodrigues et al. reported that in HT29 cells mutated p53 is

highly expressed [67]. Similar to HT29 cells, Caco-2 cells also contain a mutated p53 gene [68]. Overexpression of p53 is found in colon tumors and is associated with low chances on disease-free survival [69]. Recently, it was described that EGR1 is required for p53 tumor suppression [70]. EGR1 was the gene most induced by curcumin at the early time points. In HT29 cells treated with a mitosis-inhibiting peptide expression of EGR1 increased already after 20 minutes [71]. Therefore, also in the case of curcumin early upregulation of EGR1 could be related to growth inhibition. Interestingly, endothelial cells contrasted with HT29 cells in that induced EGR1 expression in these cells was found to be suppressed by curcumin [72]. Another interesting observation in our study is the differential expression of p16(INK4) (CDKN2A), RB1 and p53 in response to curcumin. Functions of these genes, working together in a signaling network regulating cell cycle, are often impaired in cancer cells [73]. Other interesting pathways that were influenced by curcumin are MAPK signal transduction and DNA repair.
In the study by Mariadason et al. [74] gene expression changes in SW620 cells in response to curcumin (25 μM) were measured at time points between 30 minutes and 48 hours. Of the 6253 genes analyzed by Mariadason et al. [74], 1350 genes are present in our study. In this subset, genes that were more than 1.5-fold up- or downregulated were identified and compared with our data. Several genes showed a similar response to curcumin in SW620 and HT29 cells (i.e. up- or downregulation in both cell types), for example activating transcription factor 4 (ATF4; up), vascular endothelial growth factor (VEGF; up), proteasome subunit PSMD8 (up), glutathione reductase (GSR; up), adenosine kinase (ADK; up), RAN binding protein 2-like 1 (RANBP2L1; down), prostaglandin D synthase (PTGDS; up), signal sequence receptor alpha (SSR1; down), ribonuclease 4 (RNASE4; down). Although both studies used colon cancer cells, there were also differences in response to curcumin between SW620 and HT29 cells. However, the fact that cell lines differ in their response to a food component is not surprising, since we found considerable differences between twelve human colon cancer cell lines when their RNA expression profiles under standard optimal culture conditions were compared (chapter 4). Moreover, the fact that SW620 colon cancer cells were derived from a metastasis of colon cancer, whereas HT29 cells were derived from a primary colon tumor, implies that substantial physiological differences exist between these cell lines, which could contribute to the differences in gene expression profile in response to curcumin. In our study expression of fewer genes was changed in Caco-2 cells than in HT29 cells after short-term exposure to curcumin. Consistent with this observation, it was reported earlier that HT29 cells are more sensitive to curcumin than Caco-2 cells [75].

Curcumin is an anti-inflammatory agent and can act as a natural non-steroidal anti-inflammatory drug (NSAID). Some of the genes differentially expressed in response to curcumin in our study were also differentially expressed in human colon cancer cells lines in

response to other non-steroidal compounds with anti-inflammatory action (NSAIDs) such as aspirin or sulindac [76]. For example, expression of ASNS, ATF4 and MTHFD2 was upregulated by sulindac and expression of BUB1B and PDCD2 was downregulated by aspirin. Similar changes in gene expression were found in our study in response to curcumin. In a microarray study with rat colon carcinoma cells, it was found that a large proportion of the genes differentially expressed in response to aspirin were also differentially expressed in the same direction in response to butyrate [27]. In invasive human colonocytes butyrate inhibited urokinase plasminogen activator (uPA) activity, and downregulated PCNA and TP53 levels after exposure for 12-18 hours [77]. In our study a similar response was found, as curcumin reduced expression of PCNA, TP53 and PLAU (uPA). This indicates that there may be some overlap in response and in mechanism of action between different NSAIDs like curcumin, aspirin and sulindac, but also between NSAIDs and butyrate. However, both aspirin and sulindac increased expression of several metallothionein genes, which were downregulated by curcumin in our study [76].

Two studies looked at the effect of curcumin and demethoxycurcumin on gene expression in human umbilical vein endothelial cells using microarrays [78, 79]. In these cells curcumin or demethoxycurcumin upregulated expression of cyclin-dependent kinase inhibitor 2D (p19INK4D) and downregulated expression of PCNA, HDAC2, MAP2K1 and PLAU. This is consistent with our results, where curcumin exposure resulted in an increased expression of cyclin-dependent kinase inhibitor 2A (CDKN2A) and a decreased expression of PCNA, HDAC1, MAP2K2 and PLAU. It is interesting to see that human cell lines derived from different origin (colon and umbilical vein) show similar responses to curcumin.

In conclusion, this study describes changes in gene expression profiles in colon cancer cells in response to exposure to curcumin and relates these gene expression changes to functional and physiological processes. To our knowledge, this is the first study that uses a genomics approach to investigate in detail the mechanisms of effects of curcumin in colon cancer cells. Studying both early and later time points allowed us to identify genes that changed in expression with time of exposure. Some known effects of curcumin were confirmed (G2/M cell cycle arrest, induction of phase-II genes) and the existing knowledge was extended with extra information (e.g. time points of the observed changes and genes involved or linked to these physiological effects). Also, potential new leads to mechanisms explaining the biological activity of curcumin were identified, for example the effect on tubulin genes and differential expression of p16(INK4)/TP53/RB1. Studying expression changes of thousands of genes has provided increased insight into the mechanism of action of curcumin in colon cancer cells, helping us to understand how this compound can protect against development of colon cancer.

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Towards an *in vitro* screening system for potential cancer-preventive compounds based on gene expression profiling of cell lines and of human colon biopsies

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Abstract

Introduction. Development of colon cancer, one of the most common forms of cancer in Western countries, is assumed to be strongly related to environmental factors, especially diet. Therefore, interest in mechanisms of colon cancer prevention by dietary factors is strong. A substantial part of the research in this area is performed with in vitro cultured colon cancer cells.

Methods. In order to select cell lines for screening of putative cancer-preventive (food) compounds, differences and similarities in gene expression profiles of 14 human cell lines derived from colonic tissue were studied using cDNA microarrays with 4000 human genes. In addition, these expression profiles of human colon cell lines were combined with gene expression profiles in biopsies of human colon tumors and normal tissue.

Results. A subset of about 450 genes varied more than four-fold in expression level across the different cell lines. Principal component analysis (PCA) was used to visualize the differences in the gene expression profiles of the panel of cell lines. Cytoskeleton genes and immune response genes are examples of functional classes of genes that contributed to the differences between the cell lines. A subset of 72 colon cancer-specific genes was identified by comparing expression profiles in human colon biopsies of tumor tissue and normal tissue. PCA analysis of expression data of the subset of colon cancer-specific genes in the cell lines resulted in a separation of the cell lines based on the tumor stage of the adenocarcinoma from which each cell line was derived. Based on these data four cell lines were chosen for ongoing studies with potential cancer-preventive agents.

Conclusions. This study indicates that this large-scale screening of cell lines can be of great value to asses utility of cell lines to screen potential chemopreventive food compounds and thus in the ongoing research into mechanisms of cancer prevention by dietary compounds.

Introduction

Colon cancer incidence is high in Western countries. In the Netherlands it is the most common form of cancer in males after prostate and lung cancer. In females it is the most common form of cancer after breast cancer [1]. During the development of colon cancer several genetic alterations (in oncogenes like K-ras and tumor suppressor genes like APC and p53) accumulate and as a consequence normal epithelium transforms into hyperproliferative epithelium, adenoma, carcinoma and eventually metastasis [2].

The process of carcinogenesis can be halted and reversed. Both prevention and induction of development of cancer can be attributed largely to environmental factors [3]. In this respect diet and dietary factors are important, especially for prevention of cancers of the gastro-intestinal tract. Willett estimated that overall between 20 and 42 percent of cancer could be avoided by dietary changes. For colon cancer this could be as high as 50 to 80 percent [4].

Food compounds can exert their preventive action at various stages of the process of development of colon cancer. Examples are protection against DNA damage by inhibition of uptake or activation of carcinogens (initiation phase) and inhibition of cell growth, induction of apoptosis or modulation of signal transduction (promotion phase). In later phases food compounds can slow down the process, e.g. suppression of invasion by inhibition of angiogenesis or effect on cell adhesion molecules (progression phase) [5, 6].

Correspondingly, interest in the mechanisms of cancer prevention by food components is strong and much research is performed in the field of prevention of colon cancer through nutritional intervention. Many mechanistic studies use colon cancer cell lines as a test system. There are many advantages in using cell lines, including availability, growth rate, homogeneous cell population and reproducibility. In addition to these practical advantages of the use of cultured cancer cells, another important advantage is a reduction of the use of laboratory animals. However, cell lines are certainly not fully representative of the tumors they were derived from, since cells are adapted to the tissue culture conditions and populations are probably enriched for fast-growing cells [7]. Furthermore, for mechanistic studies on cancer prevention not only models of colon cancer are required, but also models that represent normal epithelium or at least very early phases of pathological onset.

Many colon cancer cell lines are available for studying effects of food compounds in colon cancer. There are differences between these cell lines and obviously the choice of cell system will influence the results. For example, to study cox-2 related mechanisms, one should select cell lines that express cox-2 and possess the intact downstream mechanisms (like prostaglandin synthesis and related receptors). Nowadays, it is possible to study gene or protein expression at a large scale. The microarray technique makes it possible to measure expression of thousands of genes in one experiment. Recently, a study reported use of large-scale gene expression profiles to predict the response of colon cancer cells to chemotherapeutic agents [8].

In this study, we used the microarray technique to measure gene expression profiles in 14 human cell lines derived from colon tissue. Two cell lines were derived from normal colon and the other 12 cell lines were derived from human colon adenocarcinomas, carcinomas or metastatic sites. Functional groups of genes were identified that were differentially expressed in the panel of cell lines. Also, information on mutations in and expression of colon cancer related genes (APC, beta-catenin, p53, cox-2, c-myc, K-ras) was collected from literature to facilitate interpretation of the expression data. During the development of colon cancer mutations in APC, K-ras and p53 accumulate [2]. In addition, other genes like c-myc, cox-2 and mismatch repair genes are also involved in colon carcinogenesis [9-11]. The role of these genes in colon cancer has been described and reviewed extensively [12-18].
A subset of colon cancer specific genes was identified by measuring and comparing gene expression profiles of biopsies from human colon tumors and their corresponding normal tissue. Next, analysis focused on expression of these colon cancer specific genes (as identified by expression profiling of the colon biopsies) in the panel of colon (cancer) cell lines. Overall, differences and similarities in a panel of colon (cancer) cell lines are elucidated by comparing expression profiles, by identifying functional classes of genes that are differentially expressed and by studying expression of colon cancer specific genes in those cell lines. These data will help to assess utility of the cell lines for screening of (food) bioactives against colon cancer.

Methods

Cell culture

Human normal colon fibroblast cell line CCD841CoTr and human colorectal adenocarcinoma and carcinoma cell lines Caco-2, Colo320hsr, HCT116, HT29, LS123, LS174T, SW480, SW620, SW837, SW1417 and T84 were purchased from the ATCC (USA). HT29mtx cells were grown as a sub-population from HT29 cells as described in Lesuffleur et al. [19]. All cells were cultured in 5% CO_2 at 37ºC using medium supplemented with 10% (v/v) fetal calf serum, penicillin, streptomycin, L-glutamine, non-essential amino acids and sodium pyruvate (Life Technologies, Invitrogen, Merelbeke, Belgium) as prescribed by the ATCC. Human normal colon epithelial cell line NCM460 was purchased from Incell Corporation (LCC, San Antonio, USA) and was cultured in M300A-500 medium (also from Incell Corporation) supplemented with 10% (v/v) fetal calf serum, penicillin and streptomycin.

Cell line characteristics

Information on mutations in and expression of specific tumor suppressor genes and oncogenes was obtained from literature and from the American Type Culture Collection (www.atcc.org).

RNA isolation from cell lines

Cells (80% confluency in 75 cm^2 culture flasks) were harvested in Trizol (Life Technologies S.A., Merelbeke, Belgium). RNA was isolated according to manufacturer's protocol. RNA clean-up and DNAse digestion was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA was checked for purity and stability by gel electrophoresis and concentration was determined spectrophotometrically.

Human colon biopsies

Biopsies of colorectal tumor tissue (n=6) and of corresponding normal colon mucosa, taken at approximately 10 cm from the tumor, were sampled after partial colectomy at the University Medical Center Nijmegen. Informed consent was obtained from each patient. Patient and tumor characteristics are given in Table 4.1. The samples were snap frozen in liquid nitrogen and stored at -80ºC.

Table 4.1. Description and origin of human colon tumor biopsies

Biopsy	Origin	Localization in colon	Grade
1	female, 43 years	transversum	pT3N1M1 (D)
2	male, 48 years	ascendens	pT3N0Mx (B)
3	female, 55 years	transversum	pT3N0Mx (B)
4	male, 60 years	cecum/ascendens	pT3N0Mx (B)
5	male, 64 years	sigmoid	pT2N0Mx (A)
6	male, 64 years	rectum/sigmoid	pT3N1M1 (D)

Grading of biopsies was performed according to TNM system. In between brackets corresponding Dukes stage is added.

RNA isolation from colon biopsies

Biopsies were homogenized in Trizol (Life Technologies S.A., Merelbeke, Belgium) by ultrathurrax. RNA isolation, RNA clean-up, DNAse digestion and check for purity and stability were similar as described for the cell lines. RNA yield was determined spectrophotometrically.

Protein expression

To determine protein expression, cells were collected by trypsinization, counted and extracted in laemli buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mM beta-mercaptoethanol). Proteins were denaturated by boiling the samples for 5 minutes at 95°C and loaded on a 10% SDS-polyacrylamide gel. Electrophoresis was followed by transfer to a polyvinyidene difluoride membrane (Roche diagnostics, Almere, the Netherlands). Membranes were blocked overnight with 5% Protifar (Nutricia, Zoetermeer, the Netherlands) in Tris-buffered saline and 0.05% TWEEN-20 (TBS-T) and then immunoblotted with antibodies against cox-2 for 1 hour. After washing in TBS-T the blots were incubated with peroxidase-conjugated secondary antibody for 1 hour. Finally, the membranes were washed extensively in TBS-T and developed using enhanced chemiluminescence reagents (Pierce,

Perbio, Etten-Leur, the Netherlands) and the signals were determined by a Lumi-imager (Roche diagnostics, Almere, the Netherlands). The antibody was purchased from Santacruz (Tebu-Bio, Heerhugowaard, the Netherlands).

cDNA microarray preparation

A set of 4069 sequence-verified human cDNA clones from the I.M.A.G.E. consortium was purchased (Research Genetics, U.S.A.) as PCR products. The cDNA was amplified by PCR with forward (5'–CTG CAA GGC GAT TAA GTT GGG TAA C-3') and reverse (5'-GTG AGC GGA TAA CAA TTT CAC ACA GGA AAC AGC-3') primers. The primers contained a 5'- C6-aminolinker (Isogen Bioscience, Maarsen, The Netherlands) to facilitate cross-linking to the aldehyde-coated glass microscope slides. PCR products were checked (for presence of single product) 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 on CSS-100 silylated aldehyde glass slides (TeleChem, Sunnyvale, CA, USA) in a controlled atmosphere. Thereafter, slides were blocked with borohydride to reduce free aldehyde residues. Slides were stored in the dark and dust-free until further use.

Transcriptomics experimental design

The experimental design to measure expression profiles of the cell lines and of the colon biopsies was similar to obtain comparable data. All samples were hybridized together with an external reference sample, which was RNA derived from human placenta. Thus, for each sample expression of the genes was measured relative to expression in the reference sample. The complete set of hybridizations for both cell lines and colon biopsies was duplicated with a dye-swap to correct for dye-specific effects.

Labeling and hybridization

Total RNA (25 µg) was labeled using CyScribe first-strand cDNA labeling kit (Amersham Biosciences, Freiburg, Germany). During reverse transcription of the RNA, Cy3- or Cy5-labelled dUTP was built into the cDNA. After incubating the reaction for 1.5 h at 42 ºC, RNA was hydrolyzed by adding NaOH. After neutralization, free nucleotides were removed from the solution using AutoSeq G50 columns (Amersham Biosciences, Freiburg, Germany).

Before hybridization, Cy3- and Cy5-labelled cDNAs were mixed and human cot-1 DNA (3 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 dried down, dissolved in 30 µl Easyhyb hybridization buffer (Roche Diagnostics, Mannheim, Germany), denatured for 1.5 min at 100 ºC and incubated for 30 minutes at 42 ºC.

Before adding the hybridization mix to the slides, slides were prehybridized in prehybridization buffer (5 x SSC, 0,1 % SDS and 10 mg/ml bovine serum albumin) for 45 minutes at 42 ºC, 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 42ºC waterbath. After hybridization, slides were washed by submersion and agitation in 0.5 x SSC with 0.2% SDS and in 0.5 x SSC. Then, slides were firmly shaken in 0.2 x SSC and put on a rotation plateau for 10 min. This step was repeated once and slides were dried quickly by centrifugation at 700 rpm.
Slides were scanned with a ScanArray Express confocal laser scanner (Perkin Elmer Life Sciences, USA) and Imagene 4.0 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images.

Data analysis
Data were imported into Microsoft Excel 97 (Microsoft Corporation, USA). Spots that were flagged manually during Imagene analysis were not included in the data analysis. Microarray data from one colon tumor and from one normal colon biopsy were excluded from the data analysis due to too many flagged spots. For each spot, local background intensity was subtracted from mean signal intensity. Expression ratio was calculated by dividing background-corrected signal intensity of the sample by the background-corrected signal intensity of the reference. Expression ratios were then log transformed (base 2) and normalized per slide using an intensity-dependent method (Lowess) [20].
In the cell lines data set the 1% or 2% lowest values for background-corrected signal were replaced by the p1% or p2% value for background-corrected signal before calculation of the expression ratios. This approach was chosen to generate expression ratios for all (non-flagged) spots, to allow for a complete comparison of the cell lines. An average expression ratio was calculated of duplicate (dye swap) arrays, provided that an expression ratio was present for both arrays. Among the tested cell lines the range of expression of each gene was calculated by subtracting the lowest average expression value (minimum) from the highest average expression value (maximum). A subset of genes, which showed the largest range of expression across the cell lines, was selected to identify genes with differential expression across the cell lines. In addition, the EASE tool [21] was used to identify functionally related subgroups of genes that were more present in the subset of genes with the largest range in expression across the different cell lines, compared to the complete set of genes present on the cDNA microarray.
The ‘significance analysis of microarrays (SAM)’ tool [22] was used to assess significance of differences in expression between normal colon tissue and colon tumor tissue. A q-value (threshold for chance of false positive detection for significant genes) of 5% was defined as

threshold for significant differential expression. Only genes with signal/background ratio greater than 1.5 in more than 50% of the microarrays were admitted to significance analysis. Principal Component Analysis (PCA), a well-known pattern recognition method in the field of multivariate data analysis, was used to analyze and visualize the gene expression profiles. Data analysis was performed using the Matlab software (The MathWorks, Inc., 1984-2001) version 6.1.0.450 (R12.1). For PCA analysis the procedure from the PLS Toolbox was used (Version 2.0.1b 1999, Eigenvector Research, Inc., 1995-1999). Average expression ratios were used in the PCA analysis. Two- (or three-) dimensional plots were made, using principal component 1 and 2 (and 3) as axes. To identify the genes that contributed most to the overall differences between the cell lines, genes with the highest scores (n=25) and the lowest scores (n=25) were selected in each of the three principal components (PCs).

Results

Characteristics of the cell lines

In this study, fourteen human colon-derived cell lines were analyzed. Table 4.2 gives an overview of the origin of the cell lines. Two cell lines resemble normal colon epithelium: CCD841CoTr is derived from fetal colon and NCM460 is derived from normal colonic mucosa [23]. Of the ten cell lines directly originating from colon cancers, most were derived from adenocarcinomas. Finally, two cell lines were derived from cancer cells at metastatic sites. The SW620 cell line was derived from a lymph node metastasis in the same patient from which the SW480 cell line was derived [24]. The T84 cell line was derived from a lung metastasis of a colon carcinoma. The Dukes stage of the original tumor is not known for HT29, Caco-2, HCT116 and T84 (American Type Culture Collection, www.atcc.org).

Table 4.2. Description and origin of human colon cell lines

Cell line	Origin	Derived from	Grade of tumor
NCM460	Hispanic male, 68 years	Normal colon	NA
CCD841CoTr	Female, 21 wks gestation	Normal colon, fetal	NA
LS174T	Caucasian female, 58 years	Colon adenocarcinoma	Dukes type B
LS123	Caucasian female, 65 years	Colon adenocarcinoma	Dukes type B
SW480	Caucasian male, 50 years	Colon adenocarcinoma	Dukes type B
SW1417	Caucasian female, 53 years	Colon adenocarcinoma	Dukes type C
SW837	Caucasian male, 53 years	Colon adenocarcinoma	Dukes type C
COLO320hsr	Caucasian female, 55 years	Colon adenocarcinoma	Dukes type C
SW620	Caucasian male, 51 years	Adenocarcinoma, metastatic site	Dukes type C
HT29	Caucasian female, 44 years	Colon adenocarcinoma	-
HT29MTX	Caucasian female, 44 years	Colon adenocarcinoma	-
Caco-2	Caucasian female, 72 years	Primary colon tumor	-
HCT116	Male	Colon carcinoma	-
T84	Male, 72 years	Carcinoma, metastatic site	-

-: not known

Table 4.3. Mutations in and expression of colon cancer genes in the panel of cell lines

Cell line	APC gene	APC expr		Beta catenin gene	Beta catenin expr		P53 gene	P53 expr		Cox-2 expr		c-myc expr		Ras gene	
NCM460										+	[28]				
CCD841CoTr															
LS174T	Wt	+	[37] [40] [38]	Mut	+	[37] [38]	Wt	+	[33] [34] [25]	+	[29] [28]	+	ATCC [25]	Mut	ATCC [25] [26]
LS123															
SW480	Mut	+	[40] [38]	Wt	+	[39]	Mut	+	[33] [35] [34]	-	[29]	+	ATCC [25]	Mut	ATCC [25] [26]
SW1417	Mut		[38]	Wt	+	[39]		-	[25]			+	ATCC [25]	Mut	ATCC [25] [26]
SW837	Mut	+	[37] [38]	Wt		[38]	Mut	+	[33]	+	[30]	+	ATCC	Mut	ATCC [26]
HT29	Mut	+	[40] [38]	Wt	+	[39]	Mut	+	[33] [35] [34]	+	[29] [30] [31] [28]	+	[25]	Wt	[25] [26]
HT29mtx															
SW620	Mut	+	[37] [40]	Wt	+	[39]	Mut	+	[33]	+	[32]	+	ATCC [25]	Mut	ATCC [25] [26]
Caco-2	Mut	+	[37] [40] [38]	Wt	+	[39]	Mut			+	[29] [31] [30]			Wt	[26]
HCT116	Wt	+	[37] [40] [38]	Mut	+	[37] [38] [39]	Wt		[34]	-	[31]			Mut	ATCC [26]
COLO320hsr	Mut	+	[37] [38]	Wt	+	[39]	Mut/ Wt		[34] [36]			++	[27]	Wt	[26]
T84	Mut		[38]				Wt		[8]	-	[28]			Mut	[26]

Wt: wild-type; Mut: mutated; +: expression; -: no expression

Table 4.3 summarizes information on mutations in colon cancer-related genes and provides data on expression in the different cell lines. These colon cancer genes include adenomatous polyposis coli (APC) and beta-catenin, p53, cox-2, c-myc and ras (table 4.3).
LS174T and HCT116 differ from the other cell lines in several aspects: both cell lines have a wild-type APC and p53 gene but a mutation in the beta-catenin gene (table 4.3). In contrast, the majority of the other cell lines contain a mutated APC and p53 gene and a wild-type beta-catenin gene (table 4.3). It is unclear whether Colo320hsr cells contain mutated or wild-type p53, conflicting results were found in literature (table 4.3). Recently, it was reported that T84 cells contain wild-type p53 (table 4.3). Mutations in the p53 gene in colon cancer cell lines correlate with a high expression of the mutant protein [33]. In a study by Cottu et al. [34] LS174T and HCT116 showed microsatellite instability, whereas several other cell lines did not (SW480, HT29, COLO320hsr). LS174T and HCT116 differ in cox-2 expression. SW480, HCT116 and T84 do not express COX-2 (table 4.3). Mostly, in cell lines expressing cox-2, cox-2 activity (prostaglandin E2 synthesis) is low but inducible. Most cell lines express c-myc (table 4.3). In COLO320hsr cells the c-myc gene is amplified and expressed at a high level [41]. HT29, Caco-2 and COLO320hsr cells contain a wild-type ras gene, while in the other cell lines ras is mutated (table 4.3). No information was found in literature on mutations in or expression of the above-mentioned genes in the cell lines CCD841CoTr, LS123 and HT29mtx. All colon cancer cell lines are tumorigenic in nude mice, except for LS123 and SW1417. The normal colon cell line NCM460 was not tumorigenic [23].

Expression profiling

Expression profiles were measured in the panel of cell lines and in the human colon biopsies. The overall line of data analysis was first to identify differentially expressed genes in both the cell line panel and the set of normal and tumor colon biopsies. This was followed by applying the set of selected colon cancer-specific genes (from the set of normal and tumor colon biopsies) for further interpretation of the differences between the cell lines, in perspective of the *in vivo* situation.

Differentially expressed genes in the cell line panel

A group of differentially expressed genes was identified by calculating the range of expression of each gene in the cell line panel. Of the more than 4000 genes analyzed, about 450 genes differed more than 4-fold in their level of expression across the 14 cell lines. Within this selection approximately 70 genes differed more than 8-fold in expression. From the subset of 450 genes functional groups of genes were identified that were specifically higher or lower expressed in some cell lines. These groups of genes are described below.
Expression of several keratin genes varied to a great extent in the cell lines. Keratins are involved in the cytoskeleton and form intermediate filaments in epithelial cells. Keratin 8 (KRT8) and keratin 18 (KRT18) are partner filaments, expressed in epithelial tissues.

Expression of KRT8 and KRT18 was lower in CCD841CoTr and COLO320hsr cells than in the other cell lines. Expression of KRT8 and KRT18 was highest in HT29, HT29mtx, SW480 and SW1417 cells (figure 4.1). The expression profile of keratin 14 (KRT14) was similar to that of KRT8 and KRT18 (figure 4.1). Expression of KRT7 was, like KRT8 and KRT18, also lowest in CCD841CoTr and COLO320hsr cells. In addition, in SW1417 cells expression of KRT20 was high and in SW480 cells expression of KRT5, tubulin alpha 2 and some other genes involved in cell structure was high (data not shown).

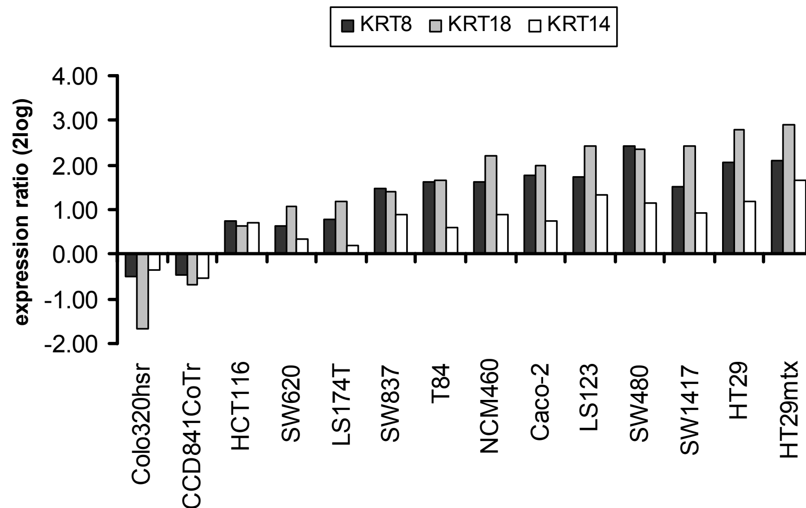

Figure 4.1. Keratin expression in 14 human colon (cancer) cell lines. KRT8: keratin 8; KRT18: keratin 18; KRT14: keratin 14. Y-axis shows ^{2}log of average expression ratio.

The gene with the largest range in expression across the cell lines is involved in cell-surface mediated signal transduction and can play a role in regulation of cell proliferation. This gene, transmembrane 4 superfamily member 4 (TM4SF4), was expressed higher in SW837, HT29 and HT29mtx cells than in the other cell lines. Moreover, expression in HT29 and HT29mtx was four-fold higher than expression in SW837 (data not shown). Another group of genes involved in cell adhesion and cell surface-mediated signal transduction are integrins. Several integrin genes were expressed at the lowest level in Caco-2 cells, e.g. integrin alpha 2 (ITGA2), integrin beta 2 (ITGB2) and integrin beta-like 1 (ITGBL1). In addition, in the total set of genes, it was observed that expression of integrin beta 4 (ITGB4), integrin beta 5 (ITGB5) and integrin beta 8 (ITGB8) was also lowest in Caco-2 cells (data not shown).

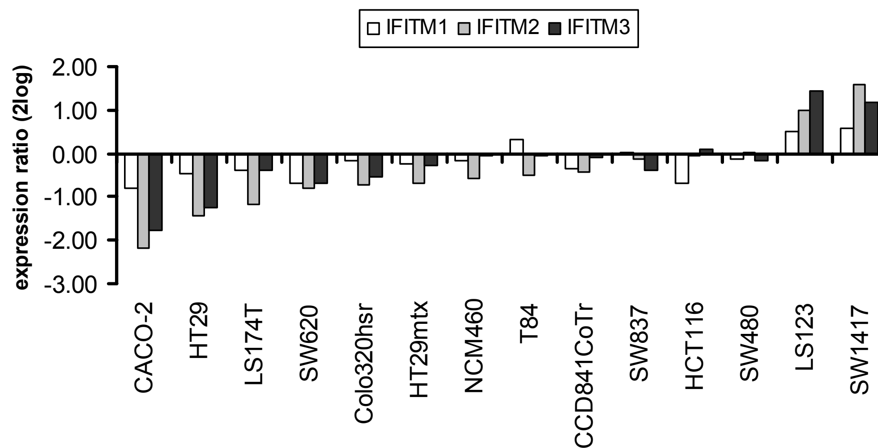

Figure 4.2. Expression of interferon-induced genes in 14 human colon (cancer) cell lines. IFITM1: interferon induced transmembrane protein 1 (9-27), IFITM2: interferon induced transmembrane protein 2 (1-8D), IFITM3: interferon induced transmembrane protein 3 (1-8U). Y-axis shows ^{2}log of average expression ratio.

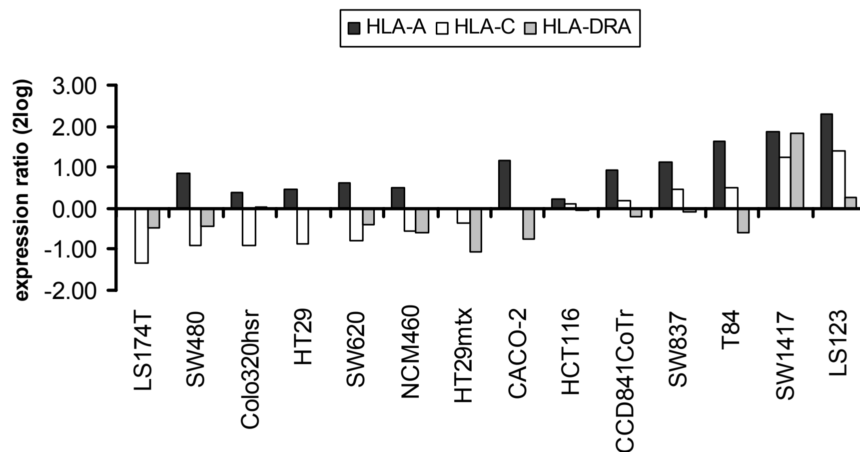

Figure 4.3. Expression of major histocompatibility complex genes in 14 human colon (cancer) cell lines. HLA-A: major histocompatibility complex, class I, A; HLA-C: major histocompatibility complex, class I, C; HLA-DRA: major histocompatibility complex, class II, DR alpha. Y-axis shows ^{2}log of average expression ratio.

A group of interferon-related genes, involved in immune response, was expressed at the highest level in SW1417 and LS123 cells (figure 4.2). This group included interferon induced transmembrane protein 1 (9-27) (IFITM1), interferon induced transmembrane protein 2 (1-8D) (IFITM2) and interferon induced transmembrane protein 3 (1-8U) (IFITM3). In the total dataset the expression of two other interferon-induced genes was also highest in SW1417 and LS123 cells, namely interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and interferon-induced protein with tetratricopeptide repeats 2 (IFIT2) (data not shown). One of the cell lines with a low expression of these immune response genes was LS174T. In this cell line three other immune response genes were expressed at the lowest level, namely three

major histocompatibility complex genes (HLA-A, HLA-C and HLA-DRA) (figure 4.3). Similar to the interferon-induced genes, expression of HLA-A, HLA-C and HLA-DRA was highest in LS123 and SW1417 cells. HLA-A and HLA-C can form a dimer with beta-2-microgloblin (B2M). This B2M gene was also included in the subset of genes with a large range of expression among the cell lines. Similar to HLA-A and HLA-C expression of B2M was highest in LS123 and SW1417 cells (data not shown).

The groups of keratin genes, of interferon-induced genes and of major histocompatibility complex genes (described above) were significantly more represented in the subset of genes with a large difference in expression (> 4-fold) compared to the total set of genes present on the microarray.

The complete set of gene expression data of the 14 cell lines was submitted to principal component analysis (PCA), allowing for grouping of cell lines with overall similar gene expression characteristics and identifying genes responsible for the overall difference between the cell lines. The three major components within the total variation between the cell lines were identified (PC1, PC2 and PC3, together explaining 37% of the total variation), yielding a three-dimensional visualization of the samples based on their expression profile (figure 4.4). The smaller the distance between the cell lines in this plot, the more similar the gene expression profiles are. In the figure different symbols are used for cell lines derived from normal colon and for cell lines derived from Dukes B or Dukes C stage colon tumors. The colon cell lines CCD841CoTr and NCM460, derived from normal tissue, plot very close to one another. Pronounced differences between these two cell lines and the cancer cell lines or between cell lines derived from different tumor stages are not found. However, with use of the information from table 4.3 several observations can be made. As was described above, the mutational profile of the cell lines LS174T and HCT116 is quite different from the other cell lines. These two cell lines plot quite close to one another in the PCA plot. The cell lines that do not express cox-2, HCT116, T84 and SW480, all have a low score on PC1 and are plotted together on the left side of the figure.

Genes that contribute most to the differences between the cell lines in the PCA plot were identified as described in materials and methods and are listed in table 4.4. The genes in the table are sorted according to function. In addition to keratin genes and immune response genes mentioned above also other genes involved in cytoskeleton and immune response, and genes involved in cell cycle, apoptosis, signal transduction, transcription regulation and transport contributed to the differences between the cell lines. Eleven genes corresponded to ESTs and are not included in the table.

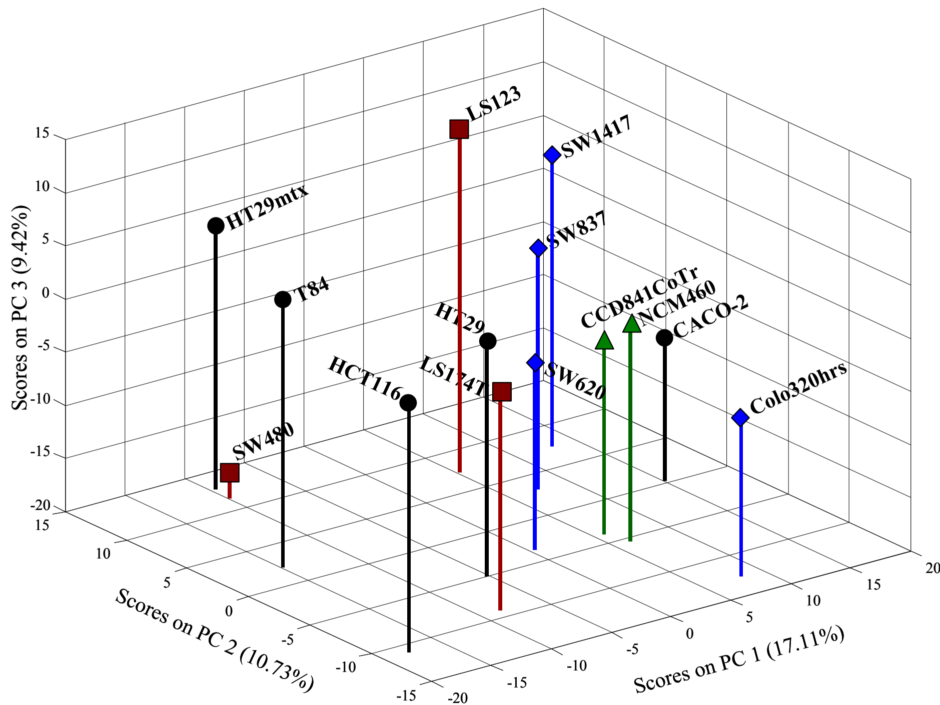

Figure 4.4. Three-dimensional visualization of Principal Component Analysis (PCA) with the total set of expression data of 14 cell lines. The gene expression pattern of each cell line is visualized as a dot in this three-dimensional graph. The axes show the scores of the gene expression profiles of the different cell lines in principal component (PC) 1, 2 and 3 (which explain the largest part of the variance). The variance explained by the PCs is indicated between parentheses.
Triangle: cell line derived from normal colon cell line; square: colon cancer cell line derived from Dukes grade B colon tumor; diamond: colon cancer cell line derived from Dukes grade C colon tumor; circles: others (information from table 4.2).

Table 4.4. Selection of genes that contribute most to differences in the panel of cell lines, based on gene expression profiles.

GenBank accession number	Gene name	Gene symbol	Function	Range of expression (fold)	lowest expression	highest expression
AA433944	Fas (TNFRSF6)-associated via death domain	FADD	apoptosis	5.9	SW1417	LS174T
N95381	APG5 autophagy 5-like (S. cerevisiae)	APG5L	apoptosis	6.7	HT29mtx	NCM460
AA454094	cullin 2	CUL2	apoptosis, cell cycle	3.7	Caco-2	HT29
AA486790	cullin 1	CUL1	apoptosis, cell cycle	3.7	HCT116	SW480
AA457038	integrin beta 1 binding protein 1	ITGB1BP1	cell adhesion	11.0	LS123	Caco-2
H78244	transmembrane 4 superfamily member 4	TM4SF4	Cell surface-mediated signal transduction, cell proliferation	50.1	T84	HT29, HT29mtx
AA464731	S100 calcium binding protein A11 (calgizzarin)	S100A11	cell cycle progression and differentiation	14.4	Colo320hsr	LS123
R19158	serine/threonine kinase 6	STK6	cell cycle regulation	5.1	HCT116	Caco-2
AA451686	cyclin C	CCNC	cell cycle regulation, transcriptional regulation	8.8	SW480	Caco-2
H84982	checkpoint suppressor 1	CHES1	cell cycle, transcription regulation	10.1	HT29	Caco-2
AA232856	topoisomerase (DNA) I	TOP1	cell growth	3.3	SW480	Caco-2
AA074222	squamous cell carcinoma antigen recognised by T cells	SART1	cell growth, immune-related?	7.7	LS123	SW480

GenBank accession number	Gene name	Gene symbol	Function	Range of expres-sion (fold)	lowest expression	highest expression
AA453749	hepatoma-derived growth factor (high-mobility group protein 1-like)	HDGF	cell proliferation	5.3	HCT116	Caco-2
N25141	cullin 3	CUL3	cell proliferation	3.7	SW1417	LS174T
AA873604	cysteine-rich protein 1 (intestinal)	CRIP1	cell proliferation, zinc ion binding	7.1	Colo320hsr	HT29mtx
AA485353	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	cell-cell interaction	4.7	NCM460	SW1417
H23255	tumor differentially expressed 1	TDE1	cellular transformation?	7.9	Colo320hsr	SW480
H08820	isopentenyl-diphosphate delta isomerase	IDI1	cholesterol synthesis	6.8	LS174T	Caco-2
H73276	actin related protein 2/3 complex, subunit 3, 21kDa	ARPC3	cytoskeleton	5.3	HT29mtx	Caco-2
AA486239	filamin B, beta (actin binding protein 278)	FLNB	cytoskeleton	8.1	SW480	NCM460
R54807	sarcoglycan, beta (43kDa dystrophin-associated glycoprotein)	SGCB	cytoskeleton	6.1	SW1417	LS174T
AA188179	actin related protein 2/3 complex, subunit 1B, 41kDa	ARPC1B	cytoskeleton	4.7	HCT116	SW480
H24708	discs, large homolog 1 (Drosophila)	DLG1	cytoskeleton	4.2	HCT116	SW480
AA449753	capping protein (actin filament) muscle Z-line, alpha 1	CAPZA1	cytoskeleton	6.8	HT29mtx	SW480
AA626698	tubulin, alpha 2	TUBA2	cytoskeleton	4.8	HCT116	SW480
AA634103	thymosin, beta 4, X-linked	TMSB4X	cytoskeleton, immune response	8.1	HCT116	LS123
AA664179	keratin 18	KRT18	cytoskeleton, intermediate filament	23.3	Colo320hsr	HT29mtx
AA485959	keratin 7	KRT7	cytoskeleton, intermediate filament	7.4	Colo320hsr	HT29mtx
AA598517	keratin 8	KRT8	cytoskeleton, intermediate filament	7.5	Colo320hsr	SW480
H44051	keratin 14 (epidermolysis bullosa simplex, Dowling-Meara, Koebner)	KRT14	cytoskeleton, intermediate filament	4.6	CCD841CoTr	HT29mtx
AA434404	primase, polypeptide 2A, 58kDa	PRIM2A	DNA replication	5.7	Colo320hsr	SW480
AA487206	glyceronephosphate O-acyltransferase	GNPAT	fatty acid metabolism	6.5	HCT116	Caco-2
N69689	RAB1A, member RAS oncogene family	RAB1A	GTP binding protein, transport	14.1	LS174T	Caco-2
R51167	RAB4A, member RAS oncogene family	RAB4A	GTP binding protein, transport	12.1	LS174T	Caco-2
W05696	RAB35, member RAS oncogene family	RAB35	GTP binding protein, transport	3.6	HT29mtx	HT29
AA085319	stanniocalcin 1	STC1	hormone activity	7.5	SW480	LS174T
R59968	crystallin, mu	CRYM	hormone binding?	3.9	Caco-2	HCT116
AA670408	beta-2-microglobulin	B2M	immune response	10.0	SW480	LS123
AA157813	interferon, alpha-inducible protein 27	IFI27	immune response	10.4	HCT116	SW837
AA644657	major histocompatibility complex, class I, A	HLA-A	immune response	5.0	LS174T	LS123
AA464246	major histocompatibility complex, class I, C	HLA-C	immune response	6.6	LS174T	LS123
AA464417	interferon induced transmembrane protein 3 (1-8U)	IFITM3	immune response	9.4	Caco-2	LS123
AA002086	CD1C antigen, c polypeptide	CD1C	immune response?	9.0	HT29mtx	SW480
AA599177	cystatin C (amyloid angiopathy and cerebral hemorrhage)	CST3	inhibitor of cysteine proteinases	3.7	LS174T	SW1417

GenBank accession number	Gene name	Gene symbol	Function	Range of expres-sion (fold)	lowest expression	highest expression
H43855	a disintegrin and metalloproteinase domain 11	ADAM11	integrin binding, cell-cell interaction	7.4	SW1417	HT29
AA485974	golgi autoantigen, golgin subfamily b, macrogolgin (with transmembrane signal), 1	GOLGB1	membrane Golgi	6.0	SW620	LS123
AA156863	phosphomannomutase 1	PMM1	metabolism	3.7	Caco-2	HCT116
AA456621	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	GGH	metabolism	5.0	HCT116	SW1417
AA668821	chitinase 3-like 2	CHI3L2	metabolism	6.2	SW480	LS123
N63940	acetylcholinesterase (YT blood group)	ACHE	metabolism	4.4	SW1417	SW480
AA280832	galactose-4-epimerase, UDP-	GALE	metabolism (carbohydrate)	4.4	LS123	LS174T
AA598510	adenine phosphoribosyltransferase	APRT	metabolism nucleoside	14.3	Caco-2	SW480
R33642	glutathione S-transferase pi	GSTP1	xenobiotic metabolism	22.9	T84	NCM460
R10662	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	MLH1	mismatch repair	4.5	SW1417	HT29mtx
AA456931	cytochrome c oxidase subunit VIc	COX6C	mitochondrial electron transport	5.4	SW480	SW1417
R71093	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	molecular chaperone, heat shock response	8.3	HT29	Caco-2
W72693	heterogeneous nuclear ribonucleoprotein A/B	HNRPAB	mRNA processing, metabolism and transport	4.1	HCT116	SW1417
H17612	arginase, type II	ARG2	nitric oxide metabolism	5.2	LS123	T84
AA598510	adenine phosphoribosyltransferase	APRT	nucleoside metabolism	14.3	Caco-2	SW480
AA423870	leucine rich repeat containing 17	LRRC17	oncogenesis?	7.1	NCM460	Caco-2
H68845	peroxiredoxin 2	PRDX2	oxidative stress	4.9	HT29	SW480
AA004759	dolichyl-phosphate mannosyltransferase polypeptide 1, catalytic subunit	DPM1	protein amino acid glycosylation	4.9	HT29mtx	HT29
AA487575	calcium and integrin binding 1 (calmyrin)	CIB1	protein binding, cell adhesion	5.7	HCT116	SW480
AA292074	ubiquitin-conjugating enzyme E2L 6	UBE2L6	protein degradation	12.0	LS174T	LS123
H65395	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	PSME2	protein degradation	4.0	HCT116	SW1417
AA486324	proteasome (prosome, macropain) activator subunit 3 (PA28 gamma; Ki)	PSME3	protein degradation	5.1	HCT116	HT29mtx
AA465237	proteasome (prosome, macropain) subunit, alpha type, 3	PSMA3	protein degradation	2.9	SW480	Caco-2
AA116060	proteasome (prosome, macropain) subunit, alpha type, 4	PSMA4	protein degradation	3.8	SW480	LS123
N38959	chaperonin containing TCP1, subunit 2 (beta)	CCT2	protein folding, molecular chaperone	20.1	SW480	HCT116
AA459351	protein phosphatase 1, regulatory subunit 7	PPP1R7	protein phosphatase type 1 regulator activity	6.2	SW480	HT29
AA521346	serine/threonine kinase 38	STK38	protein phosphorylation?	5.8	T84	HCT116
T53792	coatomer protein complex, subunit beta 2 (beta prime)	COPB2	protein transport	4.3	SW480	SW837

GenBank accession number	Gene name	Gene symbol	Function	Range of expres-sion (fold)	lowest expression	highest expression
N78621	adaptor-related protein complex 1, gamma 1 subunit	AP1G1	protein transport	5.3	HT29mtx	HT29
AA598868	coatomer protein complex, subunit beta	COPB	protein transport	9.1	SW480	HCT116
W47485	opioid receptor, sigma 1	OPRS1	receptor, immune-related?	3.7	LS174T	SW480
R60301	neurotrophic tyrosine kinase, receptor, type 2	NTRK2	receptor, signal transduction	7.9	LS174T	SW480
AA481780	carbonic anhydrase III, muscle specific	CA3	reversible hydration of carbon dioxide	7.0	SW1417	SW480
AA872341	ribosomal protein S15a	RPS15A	ribosome	3.0	SW480	Colo320hsr
R23752	mitochondrial ribosomal protein S12	MRPS12	ribosome (mitochondrial)	6.6	CCD841CoTr	SW480
AA626845	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	DDX3X	RNA binding protein	4.5	LS174T	NCM460
AA425687	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1	DDX1	RNA binding protein	3.5	T84	Caco-2
AA405000	ribonuclease T2	RNASET2	RNA catabolism	9.0	SW480	HT29
AA098980	protein kinase C-like 2	PRKCL2	signal transduction	9.3	Colo320hsr	NCM460
AA598668	chimerin (chimaerin) 1	CHN1	signal transduction	6.5	NCM460	LS174T
H07878	G protein-coupled receptor 19	GPR19	signal transduction	9.6	SW1417	HT29
AA487426	Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	signal transduction	4.8	SW480	NCM460
H70047	regulator of G-protein signalling 13	RGS13	signal transduction	5.8	SW480	HCT116
AA085319	stanniocalcin 1	STC1	signal transduction	7.5	SW480	LS174T
AA598601	insulin-like growth factor binding protein 3	IGFBP3	signal transduction, cell growth	13.0	HCT116	LS123
AA482489	interleukin 18 receptor 1	IL18R1	signal transduction, immune response	9.1	NCM460	T84
AA487614	crystallin, beta A1	CRYBA1	structural component	8.5	SW480	NCM460
H98218	high mobility group AT-hook 2	HMGA2	transcription regulation	5.2	Caco-2	HT29
T67521	U2(RNU2) small nuclear RNA auxiliary factor 1-like 2	U2AF1L2	transcription regulation	11.4	SW1417	HCT116
T58873	FOS-like antigen 2	FOSL2	transcription regulation	8.8	Caco-2	HCT116
W81685	transcription elongation factor B (SIII), polypeptide 1 (15kDa, elongin C)	TCEB1	transcription regulation	2.6	HCT116	Caco-2
N26665	suppressor of hairy wing homolog 2 (Drosophila)	SUHW2	transcription regulation	5.1	SW480	Colo320hsr
AA460265	ets variant gene 5 (ets-related molecule)	ETV5	transcription regulation	9.3	SW1417	Colo320hsr
H27986	LIM domain only 4	LMO4	transcription regulation	12.9	SW480	HT29
R08932	HMG-box transcription factor 1	HBP1	transcription regulation	9.5	SW480	LS123
AA018906	transcription factor AP-2 beta (activating enhancer binding protein 2 beta)	TFAP2B	transcription regulation	10.7	Caco-2	LS123
N59119	zinc finger protein 207	ZNF207	transcription regulation	12.4	SW480	HT29mtx
AA446018	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	SMARCB1	transcription regulation	5.8	HT29mtx	SW480
AA454926	aryl hydrocarbon receptor interacting protein	AIP	transcription regulation	14.9	HCT116	SW480
H59620	insulin induced gene 1	INSIG1	transcription regulation, cell growth	5.8	HT29mtx	SW620
AA047039	eukaryotic translation initiation factor 1A, Y-linked	EIF1AY	translation initiation	5.9	Caco-2	HCT116

GenBank accession number	Gene name	Gene symbol	Function	Range of expres-sion (fold)	lowest expression	highest expression
R93621	eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	EIF2S2	translation initiation	3.7	T84	Caco-2
H18070	mitochondrial translational initiation factor 2	MTIF2	translation initiation	42.7	LS123	Colo320hsr
AA452841	solute carrier family 12 (potassium/chloride transporters), member 4	SLC12A4	transport	6.9	SW837	HT29
H60423	solute carrier family 17 (sodium phosphate), member 2	SLC17A2	transport	8.6	Caco-2	T84
AA406552	solute carrier family 2 (facilitated glucose transporter), member 3	SLC2A3	transport	12.1	HT29mtx	Caco-2
W46972	solute carrier family 20 (phosphate transporter), member 1	SLC20A1	transport	5.1	SW480	LS174T
H78466	solute carrier family 30 (zinc transporter), member 9	SLC30A9	transport	9.5	SW480	HCT116
AA010503	N-ethylmaleimide-sensitive factor attachment protein, gamma	NAPG	transport	6.4	Caco-2	LS174T
AA126009	FXYD domain containing ion transport regulator 3	FXYD3	transport	8.3	Caco-2	LS123
AA598868	coatomer protein complex, subunit beta	COPB	transport	9.1	SW480	HCT116
AA446017	suppression of tumorigenicity 5	ST5	tumor suppressor	8.2	SW480	T84
N33574	inhibitor of growth family, member 1	ING1	tumor suppressor, cell growth	17.1	LS174T	SW480
AA459401	kallikrein 10	KLK10	tumor suppression, proteolysis	11.9	Colo320hsr	SW1417
AA454743	kallikrein 6 (neurosin, zyme)	KLK6	tumor suppression, proteolysis	6.9	CCD841CoTr	SW1417
AA451741	ilvB (bacterial acetolactate synthase)-like	ILVBL	unknown, amino acid biosynthesis?	5.9	SW1417	T84
H45300	NEL-like 2 (chicken)	NELL2	unknown, cell growth regulation?	7.6	Caco-2	SW480
R53998	CBF1 interacting corepressor	CIR	unknown	8.7	HT29	SW480
AA129397	deleted in azoospermia 4	DAZ4	unknown	7.2	Caco-2	HCT116
AA449975	dynactin 6	DCTN6	unknown	4.6	LS123	HT29
AA455925	four and a half LIM domains 1	FHL1	unknown	3.4	HT29mtx	SW837
AA113339	FSHD region gene 1	FRG1	unknown	5.5	SW1417	Caco-2
AA455303	growth factor, augmenter of liver regeneration (ERV1 homolog, S. cerevisiae)	GFER	unknown	8.2	Colo320hsr	T84
N62562	intraflagellar transport protein IFT20	LOC90410	unknown	7.5	HCT116	SW480
AA609976	mitochondrial capsule selenoprotein	MCSP	unknown	8.9	T84	SW480
AA430035	reticulon 3	RTN3	unknown	6.6	SW480	HT29
AA481464	sorting nexin 22	SNX22	unknown	3.7	LS123	HCT116

Expression of human colon cancer-specific genes in the cell line panel

Expression profiles of human colon biopsies of tumor tissue were compared to expression profiles of normal tissue. Table 4.5A shows 52 genes that were significantly higher expressed in colon tumor tissue compared to normal colon tissue. A large proportion of these genes were involved in the process of gene transcription, translation and protein synthesis. In addition, 20 genes were expressed at a significantly lower level in colon tumor tissue than in normal colon

tissue (table 4.5B). These included several metallothionein genes and two carbonic anhydrases.

Eleven genes were identified that were both significantly differentially expressed in colon tumor tissue versus normal colon tissue and showed an greater than 4-fold range in expression across the different human colon (cancer) cell lines. These genes are listed in table 4.6, together with the three cell lines with the highest and lowest expression of these genes. Expression of c-myc was highest in COLO320hsr and expression in this cell line was about 2-fold higher in COLO320hsr cells than in SW620 cells, which had the second highest expression of c-myc.

C-myc was the only gene of the colon cancer genes described in table 4.3 that was significantly differentially expressed in colon tumor biopsies compared to normal colon biopsies. The other tumor suppressor and oncogenes discussed in table 4.3 (APC, beta-catenin, p53, cox-2 and ras) were also not included in the subset of genes with more than four-fold variation in expression among the cell lines or in the list of genes that contribute most to the differences between the cell lines. However, in figure 4.4 it was observed that cell lines that do not express cox-2, plot closely together. Therefore, the cell lines were divided in three groups based on cox-2 expression, prior to principal component analysis together with the expression profiles of human biopsies of normal colon and of colon tumors (figure 4.5A). Caco-2 and NCM460 cells were grouped together, expressing high protein levels of cox-2 (figure 4.5B). HCT116, T84 and HCT116, not expressing cox-2 (table 4.3), were also grouped together. The third group consisted of the remaining cell lines. Figure 4.5A shows a clear separation of the 'no cox-2' cell lines and the 'high cox-2' cell lines and, interestingly, the normal colon biopsies are projected together with the cell lines that do not express cox-2 in the lower part of the PCA figure and the tumor tissue biopsies are projected together with the cell lines with high expression of cox-2 in the upper part of the PCA figure.

To further compare expression profiles of colon biopsies and colon cell lines, another principle component analysis of the expression data of the cell lines was performed. This evaluation was not based on the complete gene expression profiles of the cell lines, but on the expression of the subset of genes that was differentially expressed in tumor compared to normal biopsies from human colon. A total of 72 genes with a significant difference in expression between human colon tumor tissue and normal human colon tissue were identified (Table 4.5A and 4.5B). This set of colon cancer-specific genes was used as a set of 'biomarker genes' in the colon cell lines comparison. Expression data of this subset of colon cancer-specific genes in the 14 human colon cell lines were submitted to principal component analysis. Both a three-dimensional (Figure 4.6A) and a two-dimensional (Figure 4.6B) visualization from this PCA are shown. Not only the cell lines, but also the biopsies from tumor and normal colon tissue are projected in the figure. A separation of cell lines derived from different tumor stages along the X-axis (PC1) can be seen most clearly in the two-

dimensional plot (Figure 4.6B). LS123, LS174T and SW480, all derived from tumors with Dukes stage B, are projected at the right side of the figure. Colo320hsr, SW1417, SW837 and SW620, all derived from tumors with Dukes stage C, are projected at the left side of the figure. NCM460 and CCD841CoTr, derived from normal tissue, and T84, HT29 and HT29mtx are projected in between these two sets of cell lines. The normal biopsies are projected at the right side of the figure and tumor biopsies at the left side of the figure (Figure 4.6B). In addition, the cell lines that do not express COX2 (HCT116, SW480 and T84) are all projected in the lower part of the figure (Figure 4.6B).

Table 4.5A. Upregulated genes in colon tumor tissue compared to normal colon tissue

GenBank Accession Number	Gene Name	Gene Symbol	Fold induction of gene expression	
AA857163	amphiregulin (schwannoma-derived growth factor)	AREG	2.03	growth factor
AA485376	adenosine monophosphate deaminase 2 (isoform L)	AMPD2	1.52	nucleotide metabolism
AA608548	SET translocation (myeloid leukemia-associated)	SET	1.43	DNA replication
H95392	H2A histone family, member X	H2AFX	1.51	DNA binding
AA668811	H3 histone, family 3A	H3F3A	2.07	DNA binding
AA448261	high mobility group AT-hook 1	HMGA1	1.77	DNA binding, regulation of transcription
AA683085	high-mobility group box 1	HMGB1	1.73	DNA binding
AA448667	chromobox homolog 1 (HP1 beta homolog Drosophila)	CBX1	1.45	transcription regulation
AA496628	non-metastatic cells 2, protein (NM23B) expressed in	NME2	1.52	transcription factor activity (c-myc transcription factor)
AA099534	activated RNA polymerase II transcription cofactor 4	PC4	1.45	transcription regulation [interim gene name]
AA459909	6-pyruvoyl-tetrahydropterin synthase/dimerization cofactor of hepatocyte nuclear factor 1 alpha (TCF1)	PCBD	1.30	transcription co-activator activity
AA045180	transcription elongation regulator 1 (CA150)	TCERG1	1.38	transcription co-activator activity
AA663986	fibrillarin	FBL	1.68	RNA binding
AA599116	small nuclear ribonucleoprotein polypeptides B and B1	SNRPB	1.80	RNA binding
AA678021	small nuclear ribonucleoprotein polypeptide E	SNRPE	2.08	RNA binding
AA669758	nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NPM1	1.55	RNA binding, regulator of p53
R93621	eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa	EIF2S2	1.69	protein synthesis (early steps)
AA598863	eukaryotic translation initiation factor 3, subunit 8, 110kDa	EIF3S8	1.65	protein synthesis (early steps)
AA872341	ribosomal protein S15a	RPS15a	1.70	protein biosynthesis
AA775874	ribosomal protein L18	RPL18	1.63	protein biosynthesis
AA625634	ribosomal protein L35	RPL35	1.66	protein biosynthesis
H23422	ribosomal protein L7a	RPL7A	1.71	protein biosynthesis
AA668301	ribosomal protein S16	RPS16	1.57	protein biosynthesis
AA634008	ribosomal protein S23	RPS23	1.52	protein biosynthesis
AA872690	chaperonin containing TCP1, subunit 6A (zeta 1)	CCT6A	1.34	protein folding
AA864479	proteasome (prosome, macropain)	PSMB5	1.42	protein catabolism

GenBank Accession Number	Gene Name	Gene Symbol	Fold induction of gene expression	
	subunit, beta 5			
AA251770	proteasome (prosome, macropain) 26S subunit, ATPase, 2	PSMC2	1.78	protein catabolism
AA489678	RAD23 homolog B (S. cerevisiae)	RAD23B	1.50	DNA binding, protein catabolism?
R96220	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	GNB2L1	1.45	signal transduction (also known as RACK1)
AA629897	laminin receptor 1 (ribosomal protein SA, 67kDa)	LAMR1	1.55	cell adhesion, signal transduction
AA135152	glutathione peroxidase 2 (gastrointestinal)	GPX2	1.84	oxidative stress
R33642	glutathione S-transferase pi	GSTP1	1.70	metabolism xenobiotics
AA629567	heat shock 70kDa protein 8	HSPA8	1.46	heat shock protein
AA448396	heat shock 10kDa protein 1 (chaperonin 10)	HSPE1	1.56	heat shock protein
AA464600	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	1.60	oncogene
H19201	ral guanine nucleotide dissociation stimulator	RALGDS	1.75	signal transduction
AA633901	transforming growth factor, beta-induced, 68kDa	TGFBI	2.04	cell adhesion
AA459380	isocitrate dehydrogenase 3 (NAD+) gamma	IDH3G	1.61	carbohydrate metabolism
R25419	similar to gb:M58597 ELAM-1 LIGAND FUCOSYLTRANSFERASE (HUMAN)		1.43	carbohydrate metabolism?
AA136710	glyoxalase I	GLO1	1.45	carbohydrate metabolism?
AA112105	farnesyltransferase, CAAX box, alpha	FNTA	1.41	farnesyltransferase activity
AA663981	immunoglobulin heavy constant gamma 3 (G3m marker)	IGHG3	2.39	immune response
AA504461	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR	1.45	lipid transport
AA669545	spermidine synthase	SRM	1.62	spermidine biosynthesis
AA464528	proteolipid protein 2 (colonic epithelium-enriched)	PLP2	1.76	ion transport
AA460727	adaptor-related protein complex 3, sigma 1 subunit	AP3S1	1.40	protein transporter activity
N75595	nuclear transport factor 2	NUTF2	1.56	protein transport into nucleus
AA490945	secretory carrier membrane protein 1	SCAMP1	2.04	vesicular transport
AA044059	voltage-dependent anion channel 1	VDAC1	1.76	transport
AA464238	platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	PAFAH1B3	1.84	unknown
AA599187	phosphoglycerate kinase 1	PGK1	1.62	unknown
R44822	phosphoribosyl pyrophosphate synthetase-associated protein 1	PRPSAP1	1.54	unknown

Table 4.5B. Downregulated genes in colon tumor tissue compared to normal colon tissue

GenBank Accession Number	Gene Name	Gene Symbol	Fold induction of gene expression	Function
AA775447	alpha-2-macroglobulin	A2M	-1.52	protease inhibitor
T58430	tenascin XB	TNXB	-1.66	extracellular matrix protein
AA155913	matrix Gla protein	MGP	-1.72	extracellular matrix protein
AA432063	thiosulfate sulfurtransferase (rhodanese)	TST	-1.70	metabolism
AA046067	UDP-glucose pyrophosphorylase 2	UGP2	-1.43	carbohydrate metabolism?
H46254	solute carrier family 6 (neurotransmitter transporter, GABA), member 1	SLC6A1	-1.75	transport
T53220	fatty acid binding protein 1, liver	FABP1	-2.85	lipid transport
AA405731	phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	-2.06	gluconeogenesis
T65736	selenium binding protein 1	SELENBP1	-2.25	selenium binding
AA133469	keratin 20	KRT20	-2.39	cytoskeleton
H72028	gelsolin (amyloidosis, Finnish type)	GSN	-2.40	cytoskeleton
H72723	metallothionein 1B (functional)	MT1B	-2.48	metal ion binding
T56281	metallothionein 1F (functional)	MT1F	-2.51	metal ion binding
H53340	metallothionein 1G	MT1G	-3.12	metal ion binding
AA872383	metallothionein 2A	MT2A	-2.38	metal ion binding
R93176	carbonic anhydrase I	CA1	-2.92	zinc ion binding
H23187	carbonic anhydrase II	CA2	-1.86	zinc ion binding
T70057	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu pol	IGJ	-2.99	immune
T50788	EST		-1.97	unknown
AA455925	four and a half LIM domains 1	FHL1	-2.15	unknown

Table 4.6. Colon cancer-specific genes with more than 4-fold range of expression in the cell line panel.

Accession Number	Gene Name	Gene Symbol	Tumor vs normal	3 cell lines with lowest expression[a]	3 cell lines with highest expression[b]
H72723	metallothionein 1B	MT1B	Down	Caco-2, T84, HT29	CCD841CoTr, HCT116, LS123
T56281	metallothionein 1F	MT1F	Down	T84, Caco-2, HT29	LS123, CCD841CoTr, LS174T
H53340	metallothionein 1G	MT1G	Down	HT29, T84, Caco-2	LS174T, LS123, CCD841CoTr
T65736	selenium binding protein 1	SELENBP1	Down	LS174T, LS123, HCT116	Colo320hsr, T84, SW837
H72028	gelsolin (amyloidosis, Finnish type)	GSN	Down	LS174T, T84, Colo320hsr	LS123, SW837, NCM460
AA133469	cytokeratin 20	KRT20	Down	HCT116, SW837, SW480	SW1417, HT29mtx, NCM460
AA464600	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	Up	NCM460, SW837, CCD841CoTr	Colo320hsr, SW620, SW1417
AA135152	glutathione peroxidase 2 (gastrointestinal)	GPX2	Up	Colo320hsr, LS123, SW837	HT29, HT29mtx, SW480
R33642	glutathione S-transferase pi	GSTP1	Up	T84, CCD841CoTr, Colo320hsr	SW1417, SW480, NCM460
AA633901	transforming growth factor, beta-induced, 68kDa	TGFBI	Up	SW480, Colo320hsr, LS174T	NCM460, SW837, SW1417
AA464238	platelet-activating factor acetylhydrolase, isoform Ib, gamma subunit 29kDa	PAFAH1B3	Up	SW1417, LS174T, Caco-2	LS123, SW480, SW837

[a] sorted by expression level of the gene, beginning with the cell line with the lowest level of expression (of the three cell lines)
[b] sorted by expression level of the gene, beginning with the cell line with the highest expression (of the three cell lines)

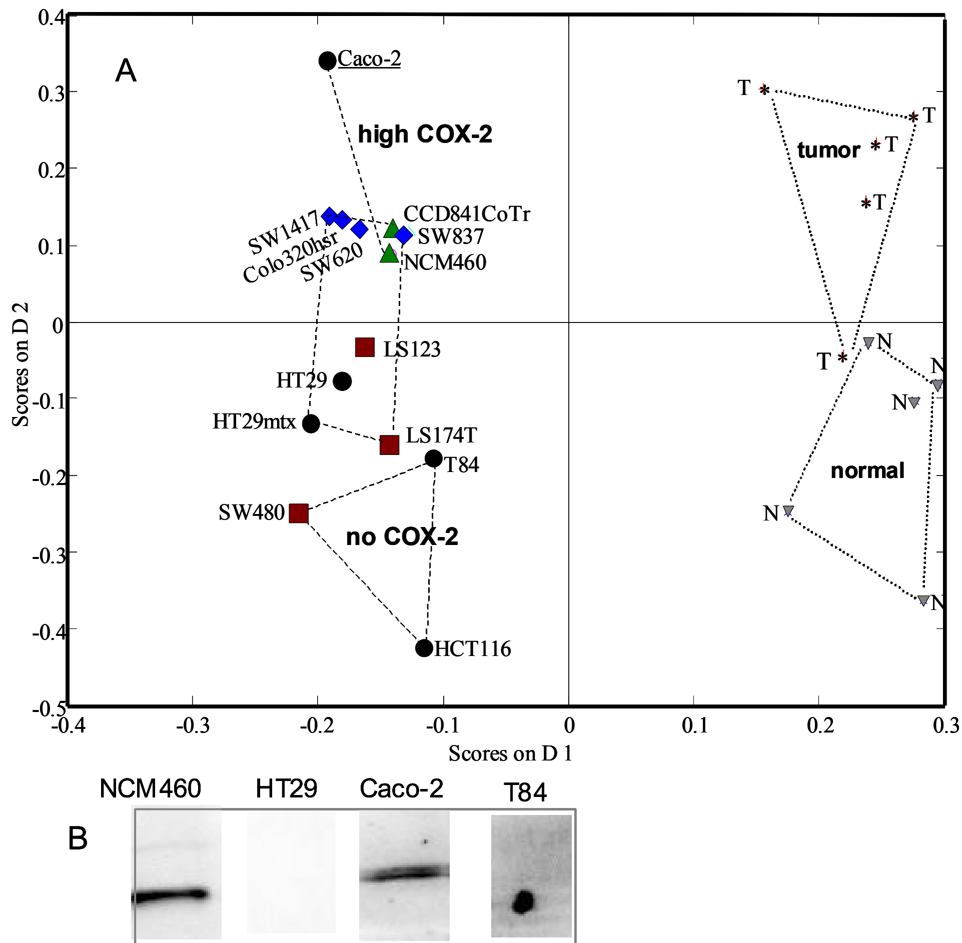

Figure 4.5. A. Principal component analysis (PCA) of expression profiles in human colon cell lines and in human colon biopsies. Prior to PCA the cell lines were divided in three groups: high COX-2 (NCM460 and Caco-2), no COX-2 (SW480, T84, HCT116) and the other cell lines (LS174T, HT29, HT29mtx, SW1417, Colo320hsr, SW620, CCD841CoTr, SW837, LS123). Human biopsies were divided in two groups: tumor tissue biopsies and normal tissue biopsies. B. COX-2 protein expression in NCM460, HT29, Caco-2 and T84 cells.

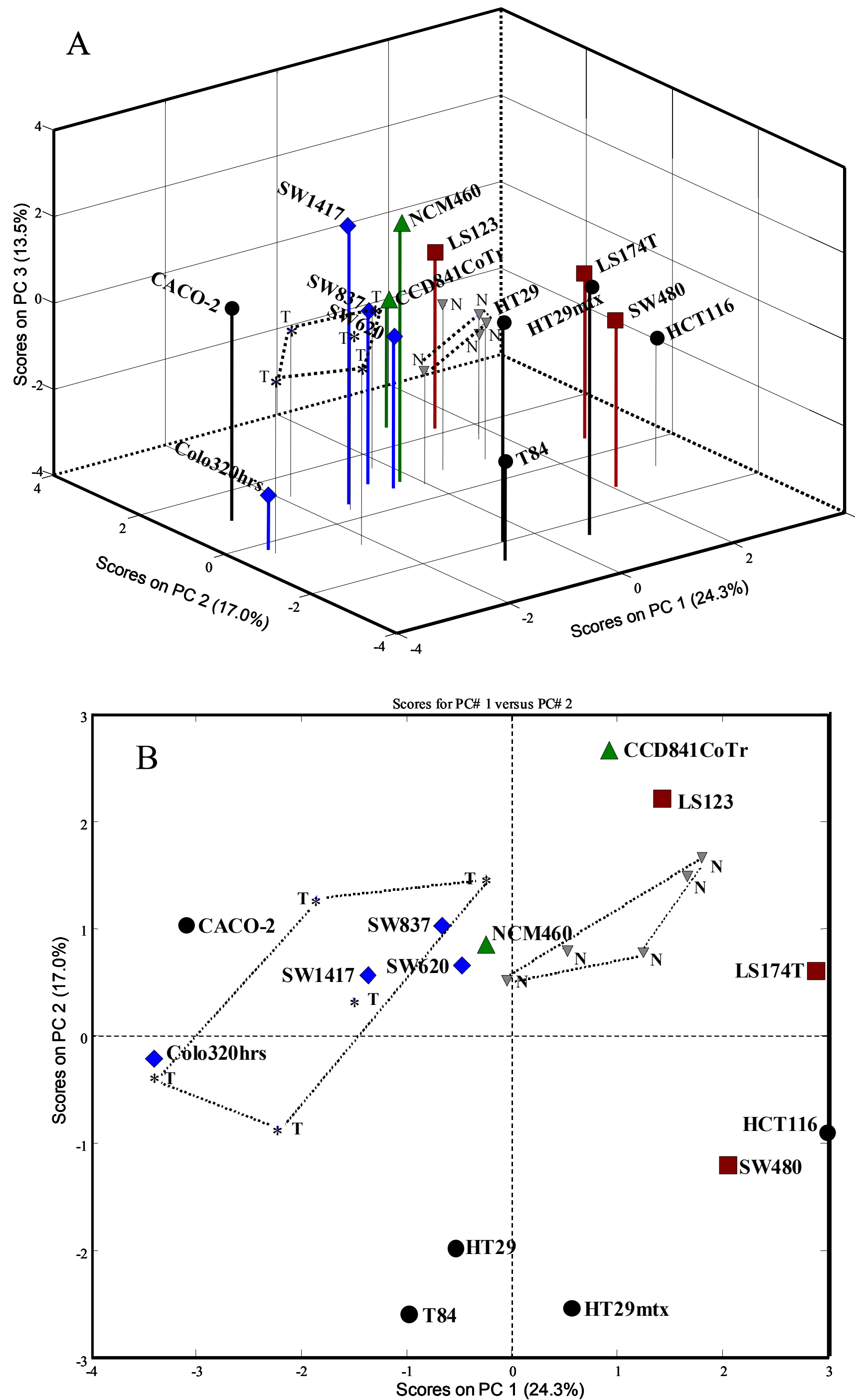

Figure 4.6. Three-dimensional (a) and two-dimensional (b) visualization of Principal Component Analysis (PCA) with expression data of 72 colon cancer specific genes in 14 human colon cell lines. The axes show the scores of the gene expression profiles of the different cell lines in principal component (PC) 1, 2 (and 3) (which explain the largest part of the variance). The variance explained by the PCs is indicated between parentheses.
Triangle: cell line derived from normal colon cell line; square: colon cancer cell line derived from Dukes grade B colon tumor; diamond: colon cancer cell line derived from Dukes grade C colon tumor; circles: others (information from table 2).
Expression profiles of biopsies are projected in the three- or two-dimensional plots. T indicates tumor colon biopsy and N indicates normal colon biopsy.

Discussion

Colon cancer cell lines are frequently used as a test system in the research into the effects of food or other bioactive compounds on colon cancer. Although all cell lines are adapted to growth outside the body, they are still considered as representative for the tissue they are derived from. Ross et al. compared gene expression profiles of 60 human cancer cell lines from different origin [42]. The main variation in expression was related to the tissue type that the cell lines were derived from, indicating that cell lines can be used as a valid tool when studying a specific type of cancer.

Although cultured cancer cells are different from cancer cells *in vivo*, molecular mechanisms underlying (anti-)cancer effects can be studied in cultured cancer cells, provided that the mechanisms are preserved. This demonstrates the importance of choosing the most adequate cell line for specific experiments, depending on the aim of the study.

For colon cancer research many cell lines are available. In this study 14 human colon (cancer) cell lines were compared with each other at the level of gene expression. Also, information on mutations in and expression of tumor suppressor genes and oncogenes was collected from literature and related to the expression data. Furthermore, *in vitro* data were compared to *in vivo* data. Therefore, gene expression profiles were measured in human biopsies of normal colon tissue and colon tumor tissue using the same experimental set-up and the same microarray design as for comparison of the cell lines.

The discussion will firstly deal with 'single gene observations' and will subsequently address the overall differences between the cell lines in respect to the *in vivo* situation, and touch upon the consequences and possibilities.

Genes that contributed to the differences between the cell lines were identified by principal component analysis of the complete expression profiles of the cell lines and by studying genes with a large (>4-fold) range of expression in the cell line panel. The principal component analysis resulted in a list of about 130 genes that contributed most to the differences between the cell lines. Functional classes of genes involved in cytoskeleton, cell surface-related signal transduction and immune responses, identified by looking at the subset of genes with a large range of expression, were also identified as differentially expressed genes in the principal component analysis. A large group of genes encoding cytoskeleton proteins was differentially expressed in the cell line panel, including keratin genes (KRT7, KRT8, KRT14, KRT18). Keratin intermediate filaments can bind both to cell membrane and nuclear membrane and can have many functions including stress resistance and apoptosis [43]. In the panel of cell lines the expression profiles of keratin 8 and 18, the most abundant keratins in intestinal tissue, was similar. Expression of these and other cytoskeleton genes was low in cell lines HCT116 and Colo320hsr and high in cell lines SW480 and HT29mtx.

Integrins and transmembrane 4 superfamily proteins are both involved in cell adhesion and in signal transduction. As part of these processes, both proteins can influence cell growth and metastasis, and it has been postulated that integrins and transmembrane 4 superfamily proteins can work together in this perspective [44]. In our study, high expression of TM4SF4 in HT29 and HT29mtx cells did not correlate with a high integrin expression.

Another group of genes that contributed to the overall differences between the cell lines and showed specific differences in expression in the panel of cell lines were immune-related genes, specifically interferon-induced genes and major histocompatibility complex (HLA) genes. Low expression of HLA-A and HLA-C in LS174T cells could be related to the microsatellite instability of this cell line, since loss of HLA and B2M expression is frequently found in colon tumors with microsatellite instability [45, 46]. Also, surface expression of the HLA-B2M complex was lower in LS174T than in SW1417 cells and a loss of expression of a specific HLA-A locus was reported for LS174T cells [47]. Expression of five interferon-induced genes (IFITM1, IFITM2, IFITM3, IFIT1, IFIT2) and expression of four major histocompatibility complex-related genes (HLA-A, HLA-C, HLA-DPA, B2M) was highest in LS123 and SW1417 cells. This observation might indicate that these two cell lines are different from the other cell lines in terms of immune-related processes and therefore that these cell lines could be useful to study immune-related effects or mechanisms. Also, these cell lines were the only two in the panel of colon cancer cell lines that are not tumorigenic.

In addition to genes involved in cytoskeleton and immune response also genes involved in cell cycle and apoptosis, signal transduction, transcription regulation, transport (mainly solute carrier family genes) and other processes were differentially expressed in the cell line panel. Several cell cycle-related genes were expressed at the highest level in Caco-2 cells. Also, a few genes that could play a role in tumor suppression were differentially expressed in the cell line panel, including kallikrein 6 and kallikrein 10.

Differences and similarities between the cell lines based on their expression profiles were visualized in a three-dimensional PCA projection (figure 4.4). The two cell lines derived from normal colonic tissue plotted close together in the three-dimensional PCA plot, indicating an overall similarity in gene expression profiles. No large distance was observed between these two cell lines and the colon cancer cell lines, indicating that the adaptation to growth outside the body has made these normal cell lines more similar to the cancer cell lines. Another observation is the distance between Colo320hsr and the other cell lines. The origin of this cell line is different from the other cell lines, namely it originates from neuroendocrine tumor cells derived from a colon carcinoma [41]. Also, the expression pattern of colon cancer-specific genes c-myc and SELENBP1 in Colo320hsr cells differed from other cell lines. Expression of these genes was considerably higher in Colo320hsr cells than in the other cell lines. High expression of c-myc is consistent with the amplification of the c-myc gene in Colo320hsr cells [27]. Besides binding of selenium, the exact function of SELENBP1 is unknown.

Consistent with a previous study [48], expression of SELENBP1 was significantly lower in colon tumor compared to normal colon tissue, indicating that this gene could play a role in colon carcinogenesis. In contrast to the high expression of SELENBP1 in Colo320hsr cells, expression of the selenoproteins GPX2, GPX1 and GPX4 was lowest in these cells compared to the other colon (cancer) cell lines (data not shown). Expression of keratins was low in Colo320hsr cells. This could be related to the fact that Colo320hsr cells grow loosely attached. Cell lines with high keratin expression, like SW480 and HT29mtx, were plotted at the opposite side of Colo320hsr in the PCA plots. This is consistent with the observation that expression level of keratin genes contributes to the differences between the cell lines.

Also, LS174T and HCT116, the two cell lines with a different profile of mutations in oncogenes and tumor suppressor genes (wild-type APC and p53, mutated beta-catenin) than the other colon cancer cell lines tested, projected close together in the PCA analysis. These two cell lines both have been reported to display microsatellite instability [34]. Gene expression data showed a high expression of metallothionein genes and a low expression of keratin genes in both cell lines. Possibly, the gene expression profiles of these cell lines are related to the mutational profile or the microsatellite instability, together indicating that these cell lines could be valuable in specific types of colon cancer research.
The three cell lines that did not express cox-2 (HCT116, T84 and SW480) were projected in the same area in the PCA plot (figure 4.4). It is known that expression of cox-2 is higher in colon tumor tissue than in normal tissue [49] and downregulation of cox-2 could be an important mechanisms of chemoprevention [17]. In contrast to what would be expected, the normal colon cell line NCM460 does express cox-2 [28] (figure 4.5B). When the cell lines were divided in three groups based on cox-2 expression prior to principal component analysis together with expression data of normal colon and colon tumor biopsies, it was observed that HCT116, SW480 and T84 (no cox-2 expression) plotted in the same area as the normal colon biopsies and that cell lines with high expression of cox-2 like Caco-2 plotted in the same area as the colon tumor biopsies. This could indicate that differences in cox-2 related mechanisms contribute to the differences between the cell lines.

In addition to this comparison of gene expression in cultured colon (cancer) cells, gene expression in human colon tumors was compared to gene expression in normal human colon tissue resulting in identification of 52 genes that were significantly upregulated and 20 genes that were significantly downregulated in colon tumors compared to normal tissue. Many of these genes were also reported to be up- or downregulated in other microarray studies comparing colon tumors with normal colon tissue. These genes included high mobility group AT-hook 1 (HMGA1) [50], several ribosomal proteins [50-52], transforming growth factor beta-induced (TGFBI) [48, 50-52], carbonic anhydrase 1 (CA1) [50, 51], carbonic anhydrase

2 (CA2) [50-53], metallothionein genes [53], fatty acid binding protein 1 (FABP1) [48, 50], selenium binding protein [48], keratin 20 [51, 53], gelsolin [51], eurkaryotic translation initiation factor 3 [50]. Many of the upregulated genes in colon tumors were involved in transcription, translation and protein synthesis, indicating that colon tumor cells could be more active in this respect.

When expression changes in the colon biopsies were compared with expression changes in the cell lines, an interesting observation was the large differences in expression between the cell lines (more than eight-fold for about 70 genes) compared to the differences in expression between normal colon tissue and colon tumor tissue, which was at most two- to three-fold. Gene expression variation between cell lines is thus much more pronounced than gene expression variation between normal and tumor tissue. This could be explained by larger variation in the biopsy data, since the data of the biopsies are an average of expression in five biopsies (each containing a mixture of cell types) while the cell lines (consisting of a single cell type) are each derived from a single tumor. The expression profiles of the cell lines differed substantially from the expression profiles of the colon biopsies, as was indicated by a clear separation between the biopsies and the cell lines in the principal component analysis with the combined set of colon biopsies data and colon (cancer) cell lines data.

The group of 72 genes with significant differences in expression between human colon tumors and normal colon tissue was used as a biomarker set of colon cancer-specific genes. This approach of identifying subsets of biomarker genes is also being applied in cancer classification, prognosis and response to treatment, e.g. in breast cancer [54] and in acute lymphoblastic leukemia [55]. In our study the subset of biomarker genes identified by expression profiling of human colon biopsies was used to relate colon cell line data to colon biopsy data. Eleven genes of the subset of colon cancer specific genes varied more than 4-fold in level of expression in the cell lines. The high level of expression of metallothionein genes in CCD841CoTr cells could reflect their normal colon tissue status. Similarly, low expression of GSTP1 and of oncogene c-myc in CCD842CoTr cells could also reflect the normal epithelium characteristics of these cells. In NCM460 cells, high expression of GSN and KRT20 and low expression of c-myc could reflect this ‘normal epithelium’ status.

The PCA analysis of the total dataset did not result in a pronounced separation of cell lines based on the tumor grade or stage of the original tumor that the cell lines were derived from. However, when the expression data of the biomarker set of colon cancer-specific genes was used in PCA analysis, a separation was observed between cell lines originating from Dukes stage B colon tumors and cell lines originating from Dukes stage C tumors. Tumor biopsies were projected in the same area as the cell lines originating from Dukes stage C tumors. Normal biopsies were projected in between the cell lines derived from normal colon tissue and the cell lines originating from Dukes stage B tumors. Caco-2 (Dukes stage unknown) was

projected close to the cell lines derived from Dukes stage C adenocarcinomas, indicating that the expression profile of the subset of colon cancer-specific genes in Caco-2 cells is similar to that in cell lines derived from Dukes stage C adenocarcinomas. HCT116 (Dukes stage unknown) was projected close to the cell lines derived from Dukes stage B adenocarcinomas, indicating that the expression profile of the subset of colon cancer-specific genes in HCT116 cells is similar to that in cell lines derived from Dukes stage B adenocarcinomas. HT29, HT29mtx and T84 were projected at equal distance from the cell lines derived from Dukes stage B and from Dukes stage C adenocarcinomas. This could indicate that the expression profile of the subset of colon cancer-specific genes in these cell lines is different from the expression profile of these genes in the cell lines derived from Dukes stage B and Dukes stage C adenocarcinomas. Similar to the PCA analysis with the complete dataset, Colo320hsr was projected distant from the other cell lines and LS174T and HCT116 were projected quite close together.
Some cell lines are abundantly used in colon cancer research, like HT29 and Caco-2, while other cell lines are only rarely reported in literature, like LS123. Correspondingly, not much is known about mutations in or expression of colon cancer related genes in the latter cell line. Obviously, an advantage of the use of often-used cell lines like Caco-2 or HT29 is comparability to other studies. In this study, microarray technology was applied to compare expression profiles of 14 human colon (cancer) cell lines, including more- as well as less-frequently used cell lines. Based on the distances between cell lines in the PCA plot of the complete expression profiles, an indication of difference or similarity of cell lines (based on gene expression data) has been obtained. Several specific gene expression characteristics that contribute to these differences were found for some cell lines, like high expression of cytoskeleton genes in SW480 cells versus low expression of cytoskeleton genes in HCT116 and Colo320hsr cells and like high expression of immune response genes in LS123 and SW1417 cells versus low expression of immune response genes in LS174T cells. Cytoskeleton genes and immune response genes are two examples of functional classes that contribute to the differences between the cell lines.

In summary, the data from this large-scale screening of cell lines can be of great value to assess utility of cell lines in the ongoing research into mechanisms of cancer prevention by dietary compounds. Considering the differences between colon cell lines it will be valuable to evaluate food compounds in more than one cell line. By studying compounds in different cell lines more information on effects and mechanisms can be collected and compared. Studies with a number of potential cancer-preventive agents are in progress. In these studies, we chose to use Caco-2, HT29, T84 and NCM460 cell lines. In the different principal component analyses performed, the colon cancer cell lines Caco-2, HT29 and T84 were plotted in different areas of the figures, indicating differences in expression profiles of these cell lines.

NCM460 cells are included as a model of normal colon tissue. The subset of 72 colon cancer-specific genes will also be used as a biomarker set in these studies. Expression changes of these genes in response to a cancer-preventive food compound will be analyzed, to observe whether these gene expression changes might reflect a shift from tumor to normal tissue, as an indication of cancer prevention. This study has pointed out interesting expression profiles of various cell lines, including less-frequently used cell lines, which will be useful for choosing the appropriate model system for all types of hypotheses in cancer prevention studies.

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5

Screening for potential cancer-preventive effects of quercetin, curcumin and resveratrol by gene expression profiling in colon cancer cell lines

IN PREPARATION

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Abstract

In a previous study expression profiles of 14 cell lines derived from colon tissue were compared. In this study, two of these cell lines (HT29 and T84) were used to screen for possible cancer-preventive effects of quercetin, curcumin and resveratrol by large-scale gene expression profiling.

The cell lines were exposed to quercetin (5 μg/ml), curcumin (10 μg/ml) or resveratrol (10 μg/ml) for 2, 6 or 24 hours. Expression changes of 17000 genes were measured using microarrays.

In general, maximal response was observed after 24 hours of exposure. All three compounds exerted a stronger effect on gene expression (larger number of genes with more than 2-fold change in expression at one or more time points) in T84 cells than in HT29 cells. Principal component analysis and functional grouping of differentially expressed genes based on Gene Ontology were used in the data analysis. Ribosomal and proteasome genes were differentially expressed in both cell lines in response to all compounds. Specifically in HT29 cells genes involved in cell cycle and genes with oxidoreductase activity were differentially expressed. Other functional groups of differentially expressed genes included DNA repair genes and genes with chaperone activity (heat shock proteins). Pronounced differences between HT29 and T84 cells were found e.g. in expression changes of tubulin and histone genes. Functional groups of differentially expressed genes are discussed in relation to potential cancer-preventive action of the compounds. In conclusion, both similar and different functional groups of differentially expressed genes were identified in HT29 and T84 cells after exposure to quercetin, curcumin and resveratrol, indicating the usefulness of using more than one model to screen for effects of potential cancer-preventive food compounds.

Introduction

Epidemiological studies suggest a strong link between diet and colon cancer [1]. Specifically consumption of fruit and vegetables is related to prevention of colon cancer [2, 3]. Consequently, many compounds present in fruit and vegetables have been identified that could play a role in colon cancer prevention. Elucidation of effects and mechanisms of action of these plant compounds is now and has been focus of many studies. Food compounds will not influence a single process in a cell, but rather exert a range of effects. Therefore, it would be more informative to study expression of thousands of genes or proteins instead of focusing on a limited selection of genes involved in (probably) known processes. Such large-scale gene expression measurements by microarray analysis can indicate which processes or pathways are affected by specific food compounds, leading to hypotheses on cancer-preventive mechanisms. In this study large-scale gene expression profiling (i.e. 17000 genes) is used to study effects in colon cancer cells of three plant compounds: quercetin, curcumin and resveratrol.

Quercetin is a flavonoid present e.g. in onions and apples and it is consumed in relatively large amounts [4]. Quercetin can inhibit proliferation *in vitro* and induce apoptosis in tumor cells [5-10]. However, quercetin has shown both cancer prevention [11, 12] and cancer promotion [13] activity *in vivo*.

Curcumin is a spice and a yellow coloring agent that is derived from the root of the plant *Curcuma longa* and is extensively used in Asian countries. Curcumin can inhibit cell proliferation and induce apoptosis in cultured colon cancer cells [14-16]. Curcumin prevented against development of cancer *in vivo*, not only in colon [13, 17] but also in stomach and duodenum [18, 19].

Resveratrol is a polyphenol that is present e.g. in the skin of grapes and therefore also in wine. In cultured cancer cells resveratrol can inhibit cell growth, induce cell cycle arrest and induce apoptosis [20-22]. In rats or mice a decreased number of tumors or preneoplastic lesions in the intestine upon administration of resveratrol was observed [23, 24]. However, recently, no effect of resveratrol was found on the number of intestinal tumors in APC(Min/+) mice [25].

In addition to effects on cell growth and apoptosis, antioxidant effects, anti-inflammatory effects and modulation of drug metabolizing enzymes are proposed to contribute to the cancer-preventive potential of quercetin, curcumin and resveratrol [26].

In this study, effects of quercetin, curcumin and resveratrol are studied in two colon cancer cells lines by large-scale gene expression profiling. In an earlier study (chapter 4 of this thesis) expression profiles of 14 cell lines derived from colon tissue were compared. It was concluded that, considering the differences between expression profiles of the cell lines, valuable additional information would be obtained when biological effects of compounds are evaluated in multiple cell lines. This chapter focuses on expression profiles in two human colon carcinoma cell lines, HT29 and T84, after exposure to quercetin, curcumin or

resveratrol. Functional groups of differentially expressed genes in HT29 and T84 cells were identified using Gene Ontology information and are discussed, including functional groups with similar responses (e.g. ribosomal genes, DNA repair genes) and functional groups with different responses (e.g. tubulin genes, histone genes) in HT29 and T84 cells.

Materials and methods

Cell culture

HT29 cells (ATCC, Rockville, USA) were grown in McCoy's 5a medium supplemented with 1.5 mM L-glutamine, 10% fetal bovine serum, penicillin and streptomycin. T84 cells (ATCC, Rockville, USA) were grown in a 1:1 mix of Ham's F12 and DMEM supplemented with 2.5 mM L-glutamine, 5% fetal bovine serum, penicillin and streptomycin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

Cells were grown in culture flaks (75 cm^2) until 80% confluency before incubation with quercetin (5 μg/ml), curcumin (10 μg/ml) or resveratrol (10 μg/ml) for 2, 6 and 24 hours. The compounds were solved in ethanol; the final ethanol concentration in the medium was 0.5%.

Protein expression

To determine protein expression, cells were collected by trypsinization, counted and extracted in laemli buffer (50 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mM beta-mercaptoethanol). Proteins were denaturated by boiling the samples for 5 minutes at 95°C and loaded on a 10% SDS-polyacrylamide gel. Electrophoresis was followed by transfer to a polyvinyidene difluoride membrane (Roche diagnostics, Almere, the Netherlands). Membranes were blocked overnight with 5% Protifar (Nutricia, Zoetermeer, the Netherlands) in Tris-buffered saline and 0.05% TWEEN-20 (TBS-T) and then immunoblotted with antibodies against p53 for 1 hour. The p53 antibody was purchased from Oncogene research products (Calbiochem, Omnilabo BV, Breda, the Netherlands). After washing in TBS-T the blots were incubated with peroxidase-conjugated secondary antibody for 1 hour. Finally, the membranes were washed extensively in TBS-T and developed using enhanced chemiluminescence reagents (Pierce, Perbio, Etten-Leur, the Netherlands) and the signals were determined by a Lumi-imager (Roche diagnostics, Almere, the Netherlands).

RNA isolation

After exposure cells were collected in Trizol (Life Technologies S.A., Merelbeke, Belgium). Total RNA was isolated from the cells according to the manufacturer's protocol. RNA clean-up and Dnase digestion was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA was checked for purity and stability by gel electrophoresis. RNA quantity was determined spectrophotometrically.

Labeling and hybridization

The RNA labeling protocol was based on the protocol 'aminoallyl labeling of RNA for microarrays' from The Institute for Genomic Research (http://www.tigr.org/tdb/microarray/ protocolsTIGR.shtml). The microarrays contained 16659 70-mer oligos (Operon, now available through Qiagen) spotted onto Corning UltraGAPS slides together with 2541 control oligos.

Total RNA (25 µg), together with control spike RNA, was reverse transcribed with Superscript II reverse transcriptase (Invitrogen Life Technologies, Breda, the Netherlands) in the presence of oligo(dT) primers and aminoallyl-dUTP (Sigma, St. Louis, MO, USA) which was built into the cDNA. This reaction mix was incubated at 42 ºC for three hours. After cDNA synthesis RNA was hydrolyzed and the resulting cDNA was purified using QIAquick spin columns (Qiagen, Hilden, Germany). The cDNA was dried down and resuspended in sodium bicarbonate buffer. Next, Cy3 or Cy5 ester was added and the reaction mixture was incubated for 1 hour to couple the Cy3- or Cy5-dye to the amino-modified cDNA. Uncoupled dye was removed from the solution by purification with the Qiagen PCR purification kit (Qiagen, Hilden, Germany).

Each sample was labeled twice, once with Cy3 and once with Cy5. cDNA from cells exposed to quercetin, curcumin or resveratrol (treated sample) was hybridized to the microarray in competition with cDNA from cells exposed to ethanol only (untreated sample), while one of the samples was labeled with Cy3 and the other with Cy5. Hybridizations were repeated with a dye swap.

Before hybridisation, Cy3- and Cy5-labeled cDNAs were mixed and human cot-1 DNA (3 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 90 µl Easyhyb hybridization buffer (Roche Diagnostics, Mannheim, Germany) and denatured for 2 minutes at 100 ºC, followed by incubation at 42 ºC for 30 minutes.

Before adding the hybridization mix to the slides, slides were prehybridized in prehybridization buffer (1% BSA, 5 x SSC, 0.1% SDS) for 45 minutes at 42 ºC, washed two times in milliQ water and dried. After pipetting the hybridization mix on the slides, the slides were covered with a plastic coverslip and hybridized overnight in a hybridization chamber (Corning, Life Sciences, Schiphol, the Netherlands) submerged in a 42ºC waterbath. After hybridization, slides were washed once by firm shaking in 1 x SSC buffer with 0.2% SDS followed by firm shaking in 0.5 x SSC buffer. The washing step was continued by firm shaking in 0.2 x SSC buffer and by shaking the tube during 10 minutes on a rotation plateau. This step was repeated once before slides were dried.

Slides were scanned with a ScanArray Express confocal laser scanner (Perkin Elmer Life Sciences, USA) and Imagene 4.0 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images, with automatic flagging of weak or negative signals and spots with non-homogeneous signal.

Data analysis

Data were imported into SAS Enterprise guide V2 (SAS Institute Inc., Cary, USA). Spots with a signal/background ratio less than 2 or spots that were flagged by the Imagene software were not included in the data analysis. For each spot, local background intensity was subtracted from mean signal intensity. The expression ratio was calculated by dividing background-corrected signal intensity of the treated sample by the background-corrected signal intensity of the untreated sample. Expression ratios were then log transformed (base 2) and normalized per slide using an intensity-dependent method (Lowess) [27]. Data were transferred to Microsoft Excel 2000 (Microsoft Corporation, USA). Expression ratios of duplicate (dye swap) arrays were combined and an average expression ratio was calculated, provided that an expression ratio was present for both arrays. The dataset with average expression ratios was used for further data analysis.

For each compound a subset of genes that were two-fold up- or downregulated at at least one of the time points was selected. Functional classes of differentially expressed genes were determined by identifying Gene Ontology categories that were significantly more represented in the subsets compared to the total set of genes present on the microarray using EASE software [28]. Gene Ontology categories with $p < 0.05$ (defined as EASE score) and a false discovery rate < 0.05 were selected.

Average expression ratios were used in the principal component analysis (PCA). PCA, a well-known pattern recognition method in the field of multivariate data analysis, was used to analyze and visualize the gene expression profiles. This data analysis was performed using the Matlab software (The MathWorks, Inc., 1984-2001) version 6.1.0.450 (R12.1). For PCA analysis the procedure from the PLS Toolbox was used (Version 2.0.1b 1999, Eigenvector Research, Inc., 1995-1999). Two- (or three-) dimensional projection plots were made, using principal component 1 and 2 (and 3) as axes. To identify the genes that contribute mostly to the overall differences between the samples, in each of the three principal components (PCs) genes with the highest scores (n=25) and the lowest scores (n=25) were selected.

Results

Changes in gene expression were measured in two colon cancer cell lines, HT29 and T84, after exposure to quercetin, curcumin or resveratrol for 2, 6 and 24 hours.

The number of genes that were expressed at at least two-fold higher or lower level in treated cells compared to untreated cells at each of the time points is shown in table 5.1. Quercetin exposure resulted in more up- or down-regulated genes in T84 cells than in HT29 cells. In both cell lines the largest response was observed after 24 hours. The number of up- and down-regulated genes after exposure to curcumin increased with time in both cell lines and was again higher in T84 than in HT29 cells. The same increase with time was seen for resveratrol in HT29 cells. In T84 cells however, exposure to resveratrol yielded the largest response after 6 hours.

Table 5.1. Number of genes differentially expressed upon exposure of HT29 or T84 cells to quercetin (A), curcumin (B) or resveratrol (C) for the indicated time period.

A. Quercetin

HT29	2h	6h	24h	T84	2h	6h	24h
> 2-fold up	83	53	191	> 2-fold up	282	354	638
> 2-fold down	86	29	84	> 2-fold down	122	109	357
total	169	82	275	total	404	463	995

B. Curcumin

HT29	2h	6h	24h	T84	2h	6h	24h
> 2-fold up	92	138	250	> 2-fold up	261	349	346
> 2-fold down	79	87	126	> 2-fold down	111	91	195
total	171	225	376	total	372	440	541

C. Resveratrol

HT29	2h	6h	24h	T84	2h	6h	24h
> 2-fold up	13	145	249	> 2-fold up	223	354	157
> 2-fold down	30	88	128	> 2-fold down	75	63	57
total	43	233	377	total	298	417	214

First, data analysis focused on functional classes of genes that were significantly more represented within the subsets of differentially expressed genes compared to the total gene set present on the microarray. These classes of genes were identified with the aid of the three Gene Ontology networks of defined terms that describe the features of a gene product (biological process, molecular function, cellular component). Table 5.2 shows these significantly overrepresented functional classes for each compound in HT29 and T84 cells.

Table 5.2. Functional classes of differentially expressed genes

HT29	% of genes (expected)[1]	Number of genes			T84	% of genes (expected)[1]	Number of genes		
QUERCETIN		2h	6h	24h	QUERCETIN		2h	6h	24h
Protein metabolism	27.3 (19.9)	30	15	57	Protein biosynthesis	6.7 (4.7)	20	29	51
					Proteasome complex	1.5 (0.4)	1	8	12
Cell cycle	10.9 (6.1)	4	12	29	Cell cycle	7.8 (6.1)	20	21	56
DNA metabolism	9.4 (4.6)	7	9	24	DNA repair	2.9 (1.6)	11	11	21
Alcohol metabolism	5.2 (1.9)	6	2	10	Chaperone activity	2.4 (1.3)	6	10	17
Structural molecule activity	11.8 (6.1)	21	8	19	RNA metabolism	4.8 (3.4)	11	17	33
Protein transport	6.1 (3.6)	7	4	12					
Oxidoreductase activity	7.3 (4.6)	8	4	16					
CURCUMIN		2h	6h	24h	CURCUMIN		2h	6h	24h
Protein metabolism	26.0 (19.9)	34	37	76	Protein metabolism	23.7 (19.9)	61	72	96
Structural molecule activity	9.8 (6.1)	25	18	20	Structural molecule activity	8.2 (6.1)	23	21	38
Nucleotide metabolism	3.7 (1.1)	4	8	11	Heat shock protein activity	0.9 (0.3)	6	4	5
RNA metabolism	6.1 (3.4)	8	12	14	Electron transport	5.0 (3.4)	18	11	16
DNA metabolism	7.7 (4.6)	3	17	31	Hexose metabolism	2.1 (0.9)	4	7	7
DNA repair	3.0 (1.6)	0	6	11					
Cell cycle	10.2 (6.1)	4	26	35					
Oxidoreductase activity	7.4 (4.6)	9	15	18					
Chaperone activity	3.7 (1.3)	3	6	11					
RESVERATROL		2h	6h	24h	RESVERATROL		2h	6h	24h
Protein metabolism	23.8 (19.9)	5	40	70	Protein metabolism	23.8 (19.9)	46	65	37
DNA repair	3.3 (1.6)	0	6	9	DNA repair	3.2 (1.6)	5	13	3
DNA metabolism	8.2 (4.6)	1	16	26	Apoptosis regulator activity	2.2 (1.0)	6	5	1
Cell cycle	9.3 (6.1)	1	19	29	Structural molecule activity	10.0 (6.1)	21	28	16
Structural molecule activity	8.8 (6.1)	6	19	25	Energy pathways	3.9 (1.8)	6	4	10
RNA metabolism (including RNA splicing)	6.5 (3.4)	1	16	15					
Oxidoreductase activity	8.8 (4.6)	4	16	25					
Chaperone activity	3.0 (1.3)	4	4	7					

Functional classes of genes (named with Gene Ontology terms) with statistically significant higher representation in the subset of differentially expressed genes than in the total set of genes present on the microarray were identified for each compound in HT29 and T84 cells.
[1]: % of total set of differentially expressed genes. Between parentheses is shown the percentage of genes that would be expected by chance in each functional class.

Expression changes in genes involved in protein metabolism and/or protein biosynthesis were found for all three compounds in both cell lines. Specifically in HT29 cells, exposure of all three compounds resulted in differential expression of genes of the functional classes cell cycle, DNA metabolism and oxidoreductase activity. Other gene ontology classes that were affected by more than one compound are chaperone activity, DNA repair and structural molecule activity.

Secondly, for each compound the complete dataset of expression changes in both HT29 and T84 cells was submitted to principal component analysis. Based on the expression profiles the three major components within the total variation were identified (PC1, PC2 and PC3), yielding a three-dimensional visualization for each of the compounds tested (Figures 5.1, 5.2 and 5.3 for quercetin, curcumin and resveratrol, respectively). For all compounds, the total variance explained by the three principal components added up to about 80% (indicating that indeed this model accounts for the large majority of the expression differences). The distance between samples in the PCA plot is an indication of similarity or dissimilarity of the gene expression profiles. Along the axis of PC1 a separation of the two cell lines is seen, most clearly for quercetin (figure 5.1) and curcumin (figure 5.2). In the figures the changes in gene expression profile in time are visualized with dotted arrows.

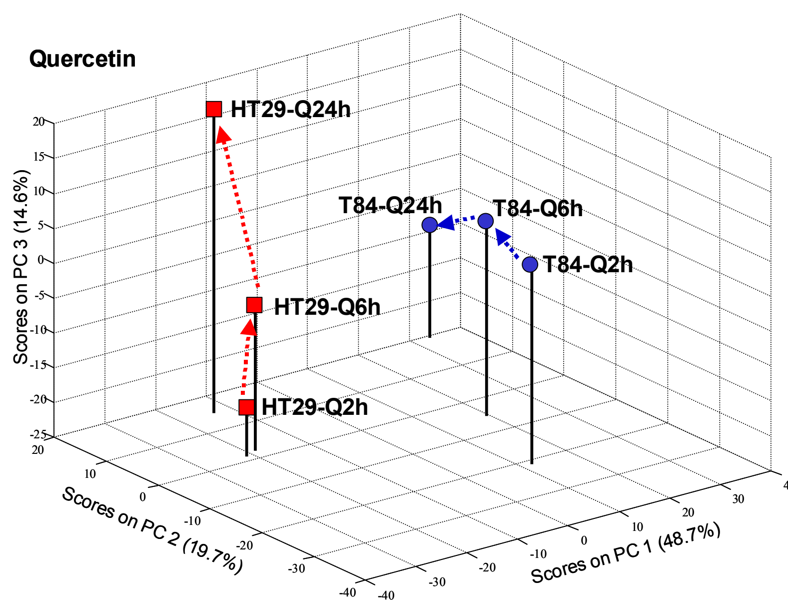

Figure 5.1. Three-dimensional visualization of the principal component analysis of the gene expression profiles of HT29 and T84 cells after exposure to quercetin (Q) for 2, 6 or 24 hours. The axes show the scores of the gene expression profiles in principal component (PC) 1, 2 and 3 (which explain the largest part of the variance). The percentage of variance explained by the PCs is indicated between parentheses.

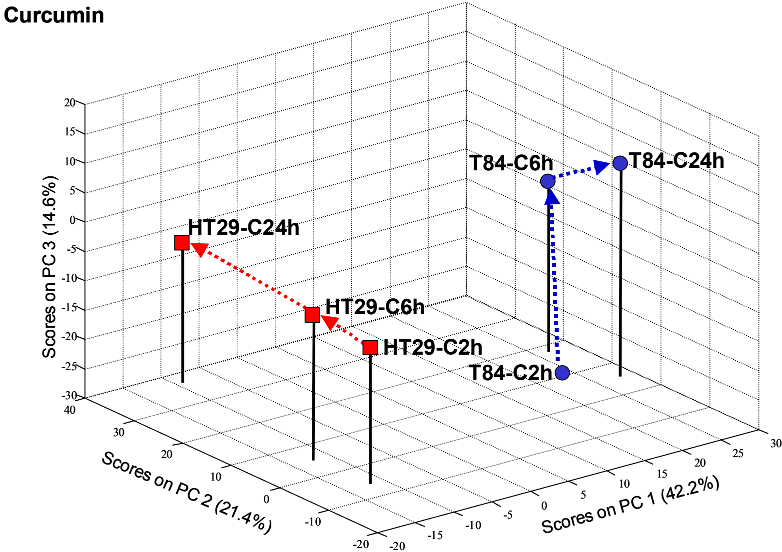

Figure 5.2. Three-dimensional visualization of the principal component analysis of the gene expression profiles of HT29 and T84 cells after exposure to curcumin (C) for 2, 6 or 24 hours. The axes show the scores of the gene expression profiles in principal component (PC) 1, 2 and 3 (which explain the largest part of the variance). The percentage of variance explained by the PCs is indicated between parentheses.

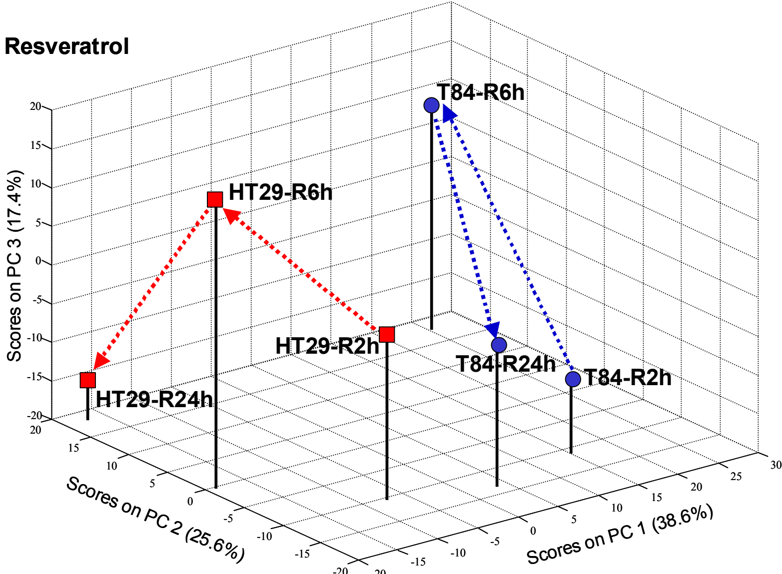

Figure 5.3. Three-dimensional visualization of the principal component analysis of the gene expression profiles of HT29 and T84 cells after exposure to resveratrol (R) for 2, 6 or 24 hours. The axes show the scores of the gene expression profiles in principal component (PC) 1, 2 and 3 (which explain the largest part of the variance). The percentage of variance explained by the PCs is indicated between parentheses.

From this principal component analysis, genes that contributed most to the differences between the samples as shown in figures 5.1-5.3 were identified (as described in materials and methods). Together, these two methods of data analysis (significance analysis of functional classes of genes based on Gene Ontology and principal component analysis) resulted in identification of functional groups of genes that were differentially expressed in colon cells after exposure to quercetin, curcumin or resveratrol. The most important functional groups are discussed in the following paragraphs.

Exposure to quercetin, curcumin and resveratrol resulted in expression changes of genes involved in protein metabolism in both cell lines, except for T84 cells exposed to quercetin (table 5.2). The protein metabolism group contained ribosomal genes involved in protein biosynthesis, genes involved in translation and proteasome genes involved in protein catabolism. Ribosomal genes and proteasome genes were also differentially expressed in T84 cells after exposure to quercetin (functional class protein biosynthesis and proteasome complex in table 5.2). When ribosomal genes and proteasome genes were considered in greater detail (table 5.3) differences between compounds and cell lines were observed. Expression of the majority of the ribosomal genes was upregulated after exposure to the compounds; only in T84 cells exposed to resveratrol several downregulated ribosomal genes were found. In HT29 cells curcumin and quercetin exposure resulted in upregulation of ribosomal genes after 2 hours. In contrast, in T84 cells ribosomal genes were mostly upregulated after 24 hours of exposure to curcumin or quercetin (table 5.3). Genes involved in translation (eukaryotic translation initiation factors) were upregulated in T84 cells exposed to quercetin or curcumin (data not shown). Expression of proteasome genes was upregulated by all compounds in both cell lines, mostly after 24 hours of exposure (table 5.3). In T84 cells less proteasome genes were upregulated after resveratrol exposure compared to quercetin or curcumin exposure (6 and 24 hours).

Table 5.3. Number of up- or down-regulated (>2-fold) ribosomal and proteasome genes in HT29 and T84 cells after exposure to quercetin, curcumin or resveratrol at different time points.

	HT29			T84		
RIBOSOMAL GENES	2h	6h	24h	2h	6h	24h
Quercetin	10 up	0	6 up 1 down	8 up 1 down	14 up 1 down	17 up 1 down
Curcumin	11 up	3 up	6 up	6 up	7 up	17 up
Resveratrol	1 up 1 down	4 up	8 up	2 up 6 down	7 up 1 down	3 up 4 down
PROTEASOME GENES	2h	6h	24h	2h	6h	24h
Quercetin	2 up	0	6 up	1 up	7 up	12 up
Curcumin	1 up	2 up	6 up	4 up	6 up	9 up
Resveratrol	0	2 up	4 up 1 down	2 up 1 down	3 up	2 up

In HT29 cells exposure to curcumin, quercetin and resveratrol resulted in an upregulation of genes involved in cell cycle, mainly after 24 hours. Curcumin exposure resulted in the largest number of differentially expressed cell cycle genes. Genes that were upregulated in HT29 cells after 24 hours in response to all three compounds included cyclin B1 (CCNB1), cyclin B2 (CCNB2), serine/threonine kinase 6 (STK6) and CDC20 cell division cycle 20 homolog (CDC20). Both up- and down-regulated cell cycle genes were found in T84 cells exposed to quercetin, e.g. upregulation of expression of cyclin-dependent kinase inhibitor 1A (p21/Cip1) after 24 hours and downregulation of two E2F transcription factors (E2F1 and E2F5) after 24 hours. In contrast to HT29 cells, no effect of curcumin and resveratrol on genes involved in cell cycle was observed in T84 cells. Resveratrol exposure for 2 or 6 hours resulted in differential expression of genes with apoptosis regulator activity in T84 cells. This group contained e.g. caspase 8 and DNA fragmentation factor alpha polypeptide (data not shown).
Genes with oxidoreductase activity were differentially expressed in HT29 cells after exposure to all three compounds. Genes within this class were e.g. NADH dehydrogenase genes and cytochrome c oxidase genes (both upregulated by all compounds). A number of cytochrome P450 genes (also in oxidoreductase activity class) were specifically downregulated by curcumin. In T84 cells exposure to curcumin resulted also in upregulation of NADH dehydrogenase genes and downregulation of cytochrome P450 genes, these were grouped in the category electron transport.
DNA repair genes were differentially expressed in HT29 cells exposed to curcumin or resveratrol and in T84 cells exposed to quercetin or resveratrol. In HT29 cells exposed to curcumin and resveratrol and in T84 cells exposed to resveratrol the majority of DNA repair genes were upregulated. However, the time of induction differed between the two cell lines. In T84 cells exposed to resveratrol DNA repair genes were mainly upregulated after exposure for 2 or 6 hours. In HT29 cells exposed to resveratrol or curcumin upregulation of DNA repair genes was only first seen after exposure for at least 6 hours and most upregulated genes were found after 24 hours. In HT29 cells expression of the mRNA encoding for p53, also involved in DNA repair, was downregulated more than 2-fold after exposure to resveratrol for 24 hours (figure 5.4). However, protein expression of p53 did not change in HT29 cells after exposure to curcumin, quercetin or resveratrol (figure 5.5). In T84 cells exposed to quercetin not only upregulated DNA repair genes but also downregulated DNA repair genes were found, e.g. mutS homolog 5 (MSH5), mutY homolog (MUTYH) or excision repair cross-complementing rodent repair deficiency 2 (ERCC2).

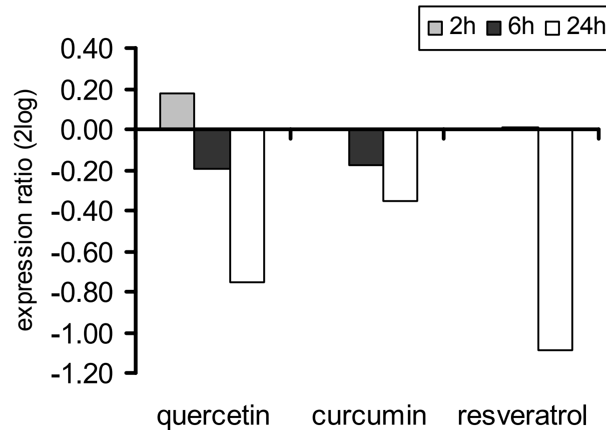

Figure 5.4. Expression changes of p53 mRNA in HT29 cells exposed to quercetin, curcumin or resveratrol as measured by microarray analysis. Y-axis shows ^{2}log values of expression ratio (level in treated cells divided by level in untreated cells).

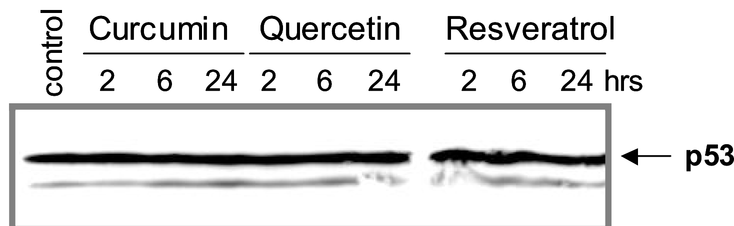

Figure 5.5. Protein expression of p53 in unexposed HT29 cells (control) and in HT29 cells exposed to curcumin, quercetin or resveratrol for 2, 6 or 24 hours.

One of the groups within the category 'chaperone activity' are the heat shock proteins. In T84 cells exposed to curcumin or quercetin gene expression of several heat shock proteins was upregulated e.g. heat shock 60 kDa protein (HSPD1), heat shock 90 kDa protein alpha (HSPCA) and a number of heat shock 70 kDa proteins. Expression of HSPCA was also upregulated in T84 cells exposed to resveratrol and in HT29 cells exposed to quercetin, curcumin or resveratrol. Expression of one heat shock protein, APG-1 (a member of the heat shock protein hsp 110 family), was downregulated in T84 cells exposed to curcumin or quercetin for 2 hours.

Genes involved in DNA metabolism were differentially expressed in HT29 cells exposed to all three compounds. A number of histone genes belong to this category. Gene expression changes of histone genes in response to quercetin, curcumin or resveratrol differed in HT29 and T84 cells (figure 5.6). In T84 cells expression of the majority of the histone genes was downregulated, however, in HT29 cells expression of the majority of the histone genes was upregulated. In contrast to most histone genes in T84 cells, expression of histone acetyltransferase 1 (HAT1) was upregulated in T84 cells exposed to quercetin (figure 5.6). Expression of histone deacetylase 2 (HDAC2) was upregulated in both cell lines in response to all three compounds (data not shown).

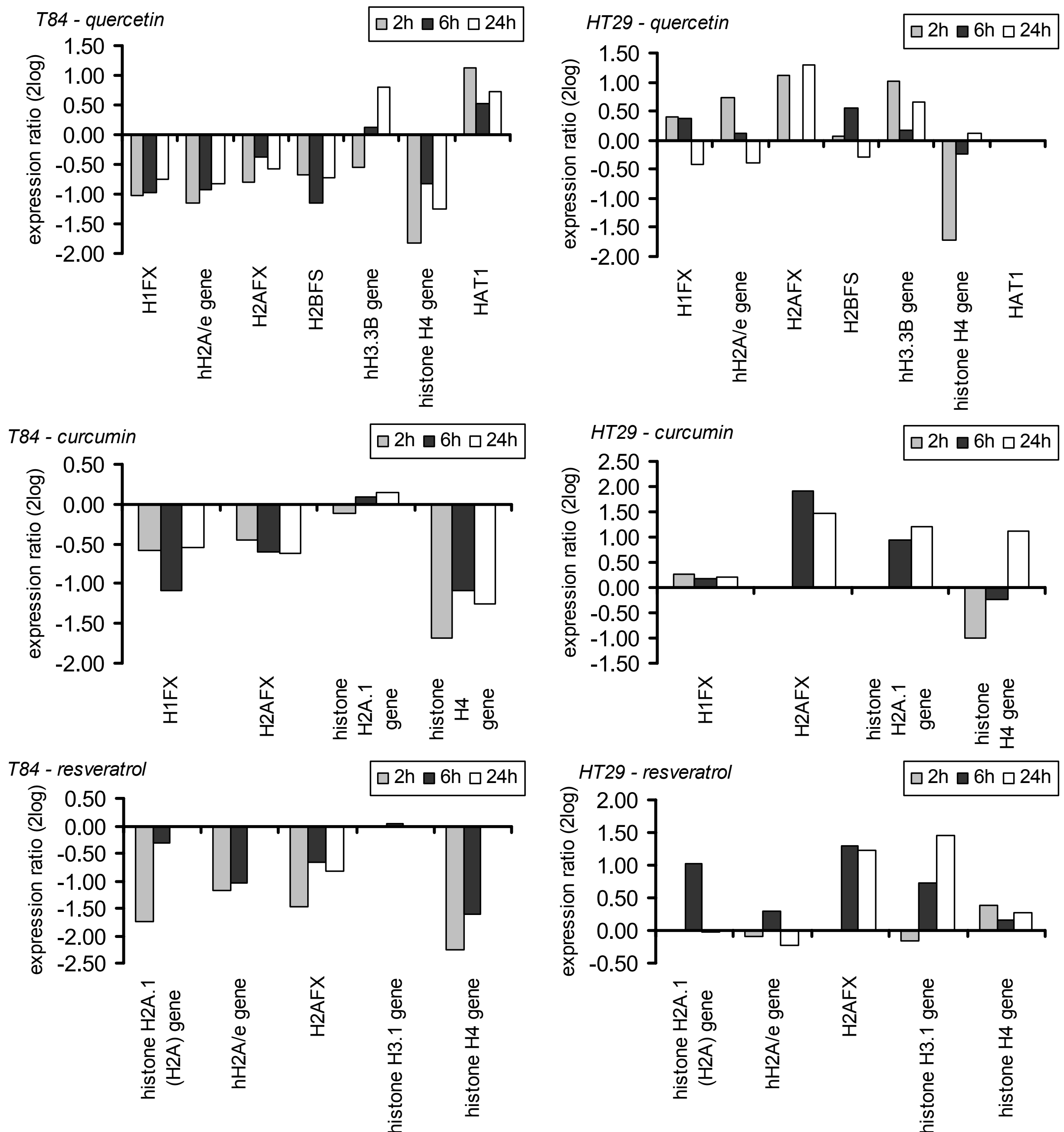

Figure 5.6. Expression changes of histone genes in T84 cells (left) and HT29 cells (right) exposed to curcumin, quercetin or resveratrol. Y-axis shows ^{2}log values of expression ratio (level in treated cells divided by level in untreated cells). H1FX: H1 histone family, member X; H2AFX: H2A histone family, member X; H2BFS: H2B histone family, member S; HAT1: histone acetyltransferase 1.

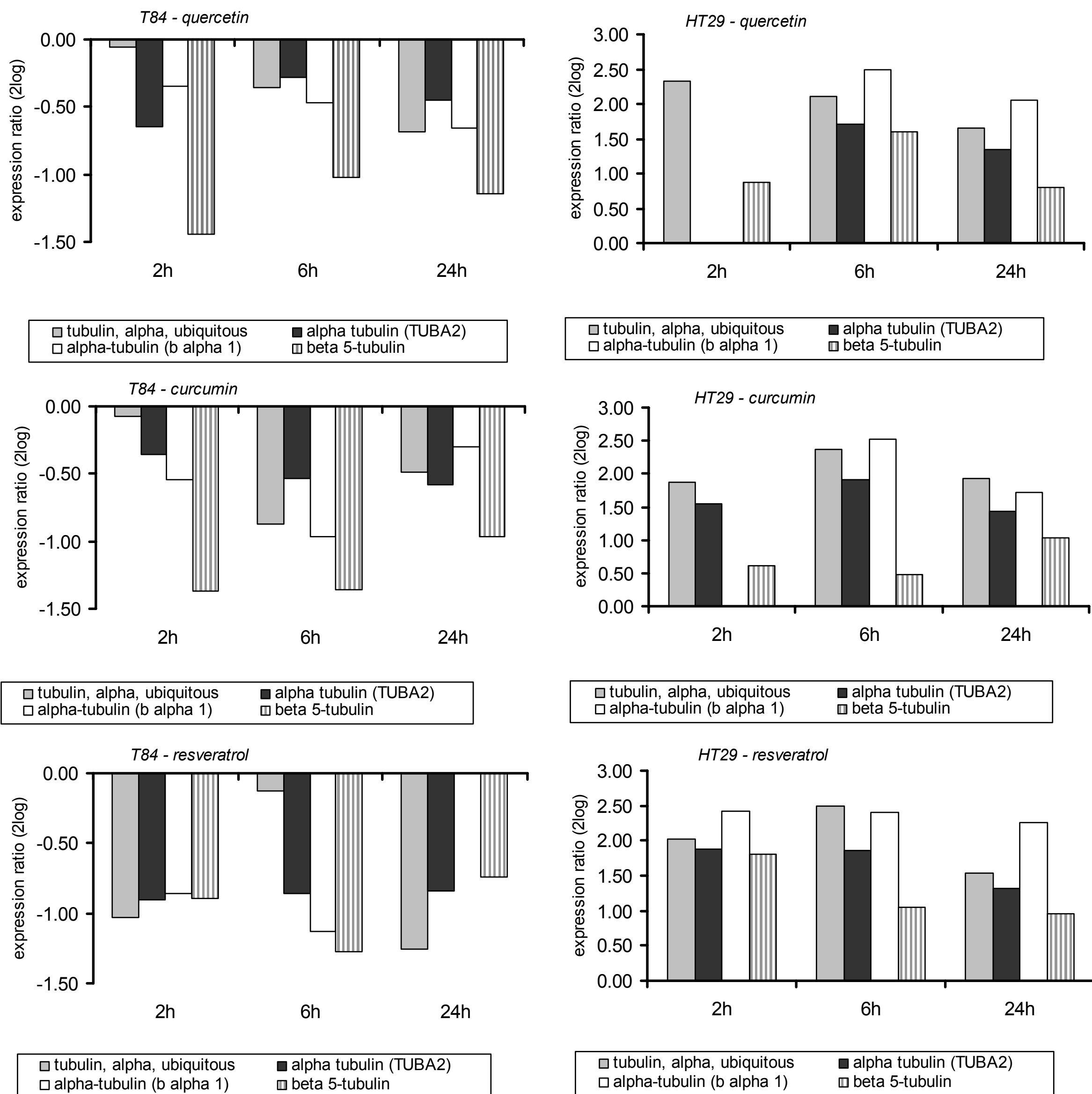

Figure 5.7. Expression changes of tubulin genes in T84 cells (left) and HT29 cells (right) exposed to quercetin, curcumin or resveratrol. Y-axis shows ^{2}log values of expression ratio (level in treated cells divided by level in untreated cells).

Another group of genes with a strikingly different response in HT29 and T84 cells were tubulin genes. Expression of tubulin genes, e.g. several forms of alpha tubulin, was downregulated in T84 cells and upregulated in HT29 cells after exposure to one of the compounds (figure 5.7). A number of single genes also show a different response to quercetin, curcumin and/or resveratrol in T84 and HT29 cells. Expression of mucin MUC5AC was upregulated in T84 cells (mainly after 2 hours) and downregulated in HT29 cells (after 2 or 6 hours) by all three compounds (data not shown). In addition, expression of two other mucins (HAB1 and MUC2) was also upregulated in T84 cells. Expression of v-myb myeloblastosis viral oncogene homolog (avian)-like 2 (MYBL2) was more than 2-fold downregulated in T84 cells exposed to quercetin (at all time points) or curcumin (after 6 hours) and more than 2-fold

upregulated in HT29 cells exposed to quercetin, curcumin or resveratrol after 24 hours (data not shown). MYBL2 is a transcription factor involved in cell cycle control. After exposure for 24 hours expression of peroxisome proliferative activated receptor gamma (PPARG) was upregulated 2-fold in T84 cells exposed to quercetin and resveratrol, but downregulated in HT29 cells exposed to quercetin (data not shown).

In a previous study a subset of 72 colon cancer-related genes was identified by comparing expression profiles of human biopsies from normal and tumor colon tissue. Expression profiles of this subset of genes in 14 human colon cell lines were submitted to principal component analysis which resulted in a separation of cell lines based on the original tumor stage that the cell line was derived from (chapter 4). In this study, the same subset of colon cancer genes was also analyzed separately to visualize shifts in the expression profiles of this subset of genes in HT29 and T84 cells after exposure to quercetin, curcumin or resveratrol. In figure 8 these shifts were visualized by plotting the expression profiles for this subset of genes as observed in the treated HT29 or T84 cells in the same PCA figure (that resulted from plotting the expression profiles observed for the subset of colon cancer-specific genes in the panel of 14 human colon cell lines). In the upper part of figure 5.8 it can be seen that the treated HT29 cells show a time-dependent shift upwards in the PCA figure in direction of both the tumor biopsies and the normal biopsies, whereas for the T84 cells, the shift was less far upward and also to the left, so that the distance towards the normal biopsies was hardly diminished upon exposure to any of the three compounds tested.

Discussion

In chapter 4 differences and similarities in expression profiles of 14 human cell lines derived from colonic tissue were studied using cDNA microarrays with 4000 human genes to assess the utility of cell lines for studying specific aspects of the mechanisms of potential cancer-prevention by food compounds. In the present study two human colon cancer cell lines (HT29 and T84) were chosen from this panel of 14 cell lines, to study effects of three plant compounds, quercetin, curcumin and resveratrol by gene expression profiling.

The origins of the two cell lines are different: T84 cells were derived from a colon cancer metastasis in the lung and HT29 cells were derived from a colon adenocarcinoma (tumor stage unknown). In addition to differences in gene expression profiles of HT29 and T84 cells, differences exist in the p53 gene (wild-type in T84 and mutated in HT29) [29, 30] and in the ras gene (wild-type in HT29 cells and mutated in T84 cells) [31]. Also, T84 cells do not express COX-2, in contrast to HT29 cells [32-34].

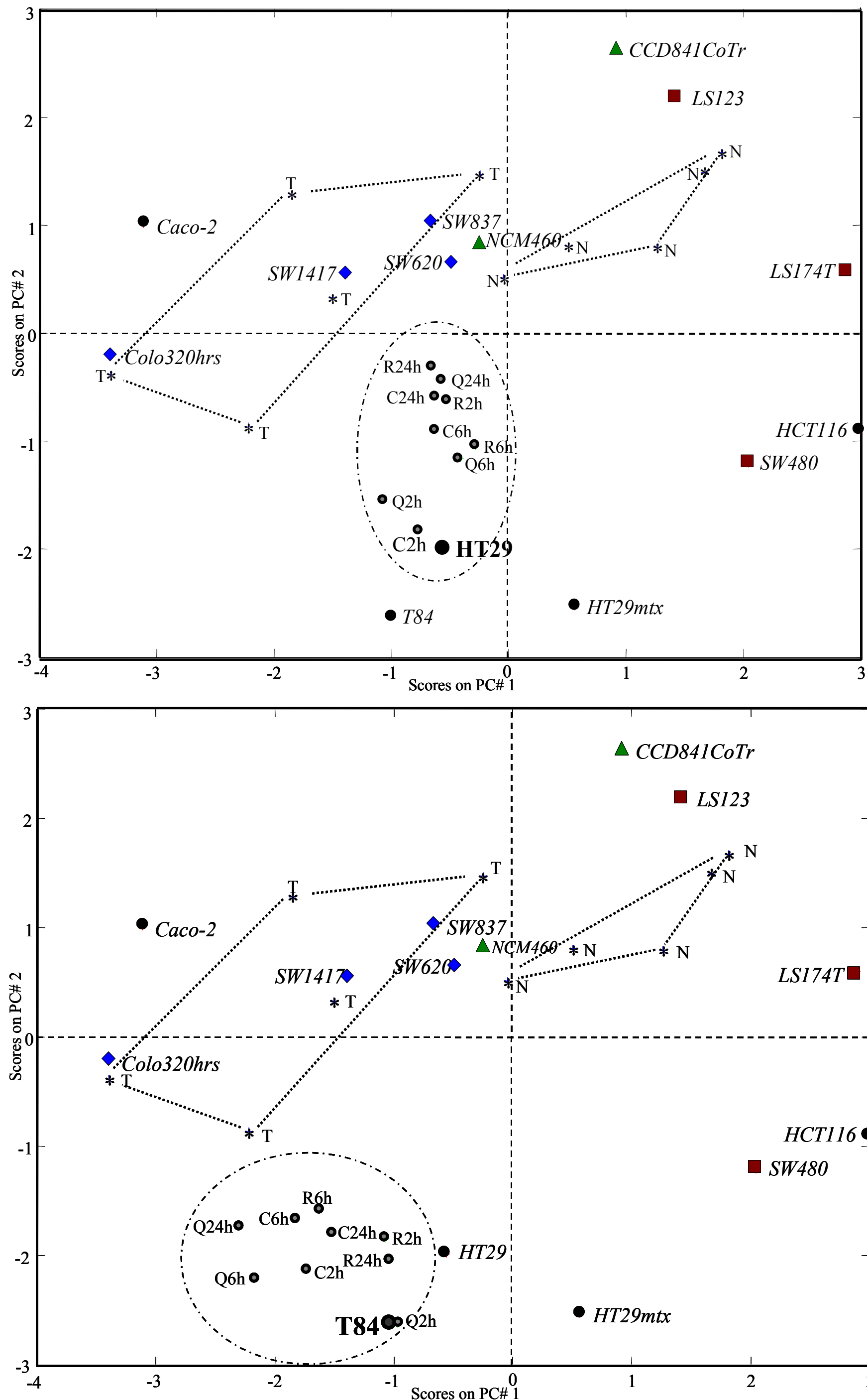

Figure 5.8. Projection of expression profile of 72 colon cancer-related genes in HT29 cells (upper) or T84 cells (lower), treated with quercetin (Q), curcumin (C) or resveratrol (R) for 2, 6 or 24 hours, in PCA figure of expression profiles of 72 colon cancer-related genes in 14 human colon cell lines (chapter 4 of this thesis). Expression profiles of biopsies (on which selection of 72 colon cancer-related genes was based) are also projected in the figure. T indicates tumor colon biopsy and N indicates normal colon biopsy.
The axes show the scores of the gene expression profiles of the different cell lines in principal component (PC) 1 and 2. Triangle: cell line derived from normal colon cell line; square: colon cancer cell line derived from Dukes grade B colon tumor; diamond: colon cancer cell line derived from Dukes grade C colon tumor; circles: others.

Functional grouping of differentially expressed genes based on Gene Ontology terms was applied to identify interesting functional groups of differentially expressed genes which can be studied in more detail. Groups of genes that responded in both cell lines to all three compounds (ribosomal genes and proteasome genes), groups of genes that responded in both cell lines or in response to more than one compound (e.g. genes with oxidoreductase activity, DNA repair genes) and groups of genes that responded specifically to one compound in one cell line (e.g. genes with apoptosis regulator activity) were identified.

Expression of ribosomal genes and of proteasome genes was induced in T84 and HT29 cells in response to all three compounds. Co-regulation of ribosomal and proteasome genes was previously found in a microarray study by Schwamborn et al. studying effects of TNF-alpha [35]. Differential expression of ribosomal genes was also reported in human colon tumors compared to normal tissue [36-38]. Upregulation of expression of proteasome genes was found in liver of mice fed a diet with a dithiolethione, belonging to a group of antioxidants and phase-II enzyme inducers [39]. This effect was not found in Nrf2-deficient mice, indicating that antioxidants could upregulate expression of proteasome genes through the Nrf2-antioxidant response element pathway [39]. Upregulation of proteasome genes could in turn lead to increased repair or degradation of damaged or oxidized proteins. In the same study, chaperone genes including heat shock genes were also identified as Nrf-2 dependent, similar to proteasome genes [40]. Heat shock proteins are linked to stress and apoptosis [41]. Altogether, upregulation of proteasome and chaperone gene expression might point to the potential of quercetin, curcumin and resveratrol to activate transcription of genes controlled by an antioxidant response element. These effects, as well as upregulation of genes in oxidoreductase activity class in HT29 cells, may be related to the antioxidant activity of the compounds. In a previous study, it was observed that curcumin upregulated expression of phase-II genes (that can also be induced through the antioxidant response element) and downregulated expression of cytochrome P450 genes in HT29 cells [42]. Downregulation of cytochrome P450 genes in response to curcumin was also found in this study.

It has been shown previously that resveratrol can induce apoptosis in cancer cells [43-45]. The present observation that resveratrol exposure resulted in differential expression of genes with apoptosis regulator activity in T84 cells supports that finding.

In addition to ribosomal and proteasome genes, also DNA repair genes were differentially expressed in both cell lines. Resveratrol exposure primarily resulted in upregulated DNA repair genes in both T84 and HT29 cells. Upregulation of expression of DNA repair genes could be a mechanism of cancer prevention, only likely to be effective however, in early stages of cancer development, before the crucial mutations become established.

It should be noted that, although the same functional groups were found among differentially expressed genes in both cell lines, there are differences, e.g. in time point of gene expression changes and also a large proportion of the differentially expressed genes in a certain

functional group in HT29 cells is different from the differentially expressed genes in the same functional group in T84 cells.

A difference in response of HT29 and T84 cells became evident in the principal component analysis (PCA), since a pronounced separation of the two cell lines was observed in the PCA figures. Genes that contribute to this difference between response in T84 cells and HT29 cells were e.g. tubulin genes and histone genes. Histones are involved in cell cycle control and histone mRNA levels are closely related to progression through the cell cycle stages [46]. In HT29 cells upregulation of cell cycle genes after 6 or 24 hours coincides with an upregulation of several histone genes at these time points, e.g. H2AFX (all compounds), histone H2A.1 gene (curcumin), histone H3.1 gene (resveratrol). Also, expression of tubulin genes was upregulated in HT29 cells in response to all three compounds. Previously, cell cycle effects of quercetin, curcumin and resveratrol have been related to inhibition of tubulin polymerization or formation of mitotic spindles [47-49]. Thus, all three compounds may affect cell cycle control in HT29 cells by differential expression of histone genes, tubulin genes and cell cycle genes. In contrast to HT29 cells, expression of a number of histone and tubulin genes was downregulated in T84 cells after exposure to quercetin, curcumin or resveratrol. Differential effects on histone and tubulin genes might be related to the origin of the two cell lines. As mentioned earlier, T84 cells were not derived directly from colon, like HT29 cells, but were derived from a metastatic site. As tubulin genes are part of the cytoskeleton they play a role in cell adhesion, a process that is involved in development of metastases. A role of tubulins in metastasis is also indicated by the observation that tubulin polymerization is affected by metastasis-associated genes [50]. Also, histone genes could be involved in the process of metastasis development. For example, Toh et al. showed that expression of metastasis-associated protein (MTA1) was closely related to acetylation level of a histone protein [51].

Based on the origin of the two cell lines, HT29 cells represent an earlier stage of carcinogenesis than T84 cells. This might be relevant since cancer-preventive compounds are more likely to exert effects in early stages of colon cancer than in later stages of colon cancer. In line with this consideration, the compounds elicited a more consistent response in HT29 cells (e.g. genes involved in cell cycle, genes with oxidoreductase activity) than in T84 cells.

This study was a continuation of a previous study in which expression profiles of a panel of 14 human colon cell lines were compared and used to select cell lines that could be used as *in vitro* screening models for potential cancer-preventive compounds. In the same study, large-scale expression profiling of human colon biopsies from normal and tumor tissue resulted in a subset of colon cancer-related genes, which could perhaps be used as a biomarker set of genes. Expression changes in this subset of genes were identified in this study. However, since a different kind of microarray was used (oligonucleotide instead of cDNA microarrays) about 10 genes of the previously identified subset were not present on the microarray used in

this study and could not be included. Expression profiles of the remaining subset of colon cancer-related genes in the treated HT29 and T84 cells were added to the PCA figure of the panel of colon cancer cell lines to visualize shifts of treated HT29 and T84 cells. Treatment of the HT29 cells resulted in a more pronounced shift from the cancer cell area of the plot towards the biopsies, including normal colon biopsies, than the T84 cells. This might illustrate the reflection made earlier that effects of food compounds are more likely to be observed in earlier stages of colon carcinogenesis. Thus use of a biomarker set of (colon cancer-related) genes identified from human colon biopsies could be useful to translate results from *in vitro* studies to the *in vivo* situation, e.g. by indicating shifts of gene expression profiles in direction of expression profiles in normal colon.

This study shows that large-scale gene expression profiling is a useful tool for studying effects and mechanisms of potentially cancer-preventive food compounds. A number of functional groups of differentially expressed genes were described in this study and discussed in relation to cancer-preventive potential of the compounds. When comparing the response in HT29 and T84 cells, both similar and different functional groups of differentially expressed genes were identified. This study points out that, for unraveling mechanisms of food compounds, using more than one cell line is a good approach since it will result in a more profound understanding of the processes and pathways affected by the tested food compounds.

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6

Do aberrant crypt foci have predictive value for the occurrence of colorectal tumors? Potential of gene expression profiling in tumors

FOOD AND CHEMICAL TOXICOLOGY, IN PRESS

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Abstract

The effects of different dietary compounds on the formation of aberrant crypt foci (ACF) and colorectal tumors and on the expression of a selection of genes were studied in rats. Azoxymethane (AOM)-treated male F344 rats were fed either a control diet or a diet containing 10% wheat bran, 0.2% curcumin, 4% rutin or 0.04% benzyl isothiocyanate for 8 months. ACF were counted after 7, 15 and 26 weeks. Tumors were scored after 26 weeks and 8 months. We found that the wheat bran and curcumin diets inhibited the development of colorectal tumors. In contrast, the rutin and benzyl isothiocyanate diets rather enhanced (although not statistically significantly) colorectal carcinogenesis. In addition, the various compounds caused different effects on the development of ACF. In most cases the number or size of ACF was not predictive for the ultimate tumor yield. The expression of a number of colon cancer-related genes was significantly different in tumors from the diet groups as compared to tumors from the control group. It was concluded that wheat bran and curcumin, as opposed to rutin and benzyl isothiocyanate, protect against development of colorectal cancer and that ACF are unsuitable as biomarker for colorectal cancer. Effects of the different dietary compounds on tissue inhibitor of metalloproteinase 1 (TIMP-1) gene expression correlated well with the effects of the dietary compounds on the ultimate tumor yield.

Introduction

The aim of the present study was to investigate whether the effects of a number of dietary compounds on the development of azoxymethane (AOM)-induced ACF and colorectal tumors correlated. In addition, the effects of these compounds on the expression of some tumor-related genes in colorectal tumors were studied.

Colorectal cancer is one of the most common causes of death from cancer in the western world. It has been generally accepted that food plays an important role in the risk of this disease. In the past decades, a vast amount of data has been obtained from laboratory animal models in which effects of food compounds on carcinogenesis were studied in carcinogen-treated rats. The merits of these widely used animal models have been proven. Nevertheless, there are some disadvantages: they are time consuming and take a considerable amount of animals and costs. Faster methods have been developed to study the effects of food components or chemicals on colorectal carcinogenesis. An example of such a method is the aberrant crypt assay. Aberrant crypt foci (ACF) are putative preneoplastic lesions that develop in the colon and rectum of carcinogen-treated rats. However, the predictive value of ACF for the development of colorectal tumors has proven to be inconsistent [1].

Colorectal cancer is the result of an accumulation of genetic changes and changes in the expression of certain genes. Investigation of these changes may provide valuable information, which can add to the understanding of carcinogenesis. Furthermore, expression levels of certain genes could be predictive of colon carcinogenesis. Therefore, in this study ACF and tumor data are combined with expression analysis of genes known to be involved in colon carcinogenesis.

In the present experiment groups of AOM-treated rats were fed a control diet or diets containing either wheat bran, curcumin, rutin or benzyl isothiocyanate. These four compounds were chosen because they have been related to an inhibitory effect on the development of colorectal cancer [2-5]. Wheat bran is the grind husk of wheat and belongs to the group of dietary fibers. By definition, dietary fiber is not susceptible to digestive enzymes in the small intestines and arrives unchanged in the large intestines where it may or may not be fermented by intestinal microflora [6]. Curcumin is a phenolic compound naturally occurring in plants and is used as a spicing and coloring agent. It is a natural non-steroidal anti-inflammatory drug (NSAID) [7, 8] and acts as an antioxidant [9, 10]. Rutin is a glycoside form of quercetin. In the large intestine the rutinol is split off by microbial enzymes, releasing quercetin [11]. Quercetin is a flavonoid, occurs naturally in plants and has antioxidative properties [12]. Benzyl isothiocyanate is one of the isothiocyanates originating from glucosinolates in cruciferous vegetables. It is an antioxidant and has cytostatic properties [13].

For expression analysis the following genes were chosen, based on reports that their expression is up- or down-regulated in colorectal cancer: carbonic anhydrase II (CA2), cyclin-dependent kinase 4 (CDK4), cyclooxygenase 2 (COX-2), cytochrome P450 1A1 (CYP1A1),

K-ras, p53, p27 and tissue inhibitor of metalloproteinase 1 (TIMP-1). Expression of CA2 was found to be down-regulated in colon tumors [14, 15]. CDK4 is involved in cell cycle regulation and is a catalytic subunit of cyclin D1. COX-2 expression is up-regulated in colon tumors and metastases. Down-regulation of COX-2 could be important in protection against development and progression of colorectal cancer [16, 17]. CYP1A1, involved in metabolism of xenobiotics, is expressed at a higher level in colon adenomas and carcinomas than in normal mucosa [18]. The oncogene K-ras is activated by mutation, which can lead to an increase in cell proliferation [19]. P53 is a transcription factor that has a role in cell cycle control [20]. Overexpression of p53 is found in colon tumors and is associated with lower disease-free survival [21]. The genes K-ras and p53 are often mutated during development of colorectal cancer; however, mutations in both genes are rare [22]. p27 is a cyclin-dependent kinase inhibitor, which can regulate cyclin D-cdk4/6 activity [23]. In rat colorectal tumors p27 levels were decreased compared to normal mucosa [24]. TIMP-1 levels were higher in plasma from patients with colon cancer [25] and gene expression of TIMP-1 was higher in colon tumors and liver metastases compared to corresponding normal tissue [26]. Additionally, TIMP-1 gene expression was higher in colon tissue from patients with metastases than from patients without metastases [25], suggesting that TIMP-1 could be involved in tumor invasion.

Materials and methods

Animals and diets

Two hundred and fourty male specific-pathogen free Fischer 344 rats (Charles River Deutschland, Sulzfeld, Germany), four weeks old, were divided into 5 groups of 48 animals each. The control group was fed an AIN^{93}-based diet. The other four groups were fed similar diets supplemented with either 10% (w/w) wheat bran (Meneba Feed Ingredients, Rotterdam, The Netherlands), 0.2% (w/w) curcumin (Fisher Scientific BV, ‘s-Hertogenbosch, The Netherlands), 4% (w/w) rutin (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 0.04% (w/w) benzyl isothiocyanate (Sigma Aldrich, Zwijndrecht, The Netherlands). 4% rutin is approximately equimolecular to 2% quercetin [2]. The supplements were added to the diets at the expense of wheat starch. The dietary levels of the supplements were in the range of effective levels described in literature.

Treatment and housing

All animals were treated with 3 weekly subcutaneous injections with AOM (Sigma-Aldrich, Zwijndrecht, The Netherlands), 15 mg/kg body weight. The first injection was given one week after the start of the experiment. The animals were housed in macrolon cages with bedding, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10 per hour.

Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 hours light, 12 hours dark.

In-life measurements

Food intake and body weight of all animals were recorded weekly during the first 3 months of the study and monthly thereafter. The animals were checked for clinical signs regularly. Animals showing a poor health condition were killed by decapitation under O_2/CO_2 anaesthesia. Necropsy was performed on these animals and on those that were found dead.

Necropsy, histology and histopathology

Seven and fifteen weeks after the start of the experiment 5 animals per group were killed by decapitation under O_2/CO_2 anaesthesia. The colon was removed, cut open longitudinally and fixed flat between filtration paper in 70% ethanol.

Twenty-six weeks after the start of the experiment 9 animals per group were killed and the same procedure as described above was followed. In addition, colon tumors were collected if present. Eight months after the start of the experiment the remaining animals were killed. The colon was removed, rinsed with 70% ethanol and examined for the presence of neoplastic changes. The number, size, and location (distance from the anus) of all colorectal tumors were recorded. Large tumors (≥ 5 mm) were cut in half; the part (with the stalk, if present) attached to the colon was covered with Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen and stored at –80 ˚C. The other half was covered with RNA-Later (Ambion, Austin, Texas, USA) and stored at –80 ˚C. RNA-Later was used to prevent breakdown of RNA. Small tumors (< 5 mm) were sampled and covered with either Tissue-Tek or RNA-Later and stored at –80 ˚C. Remaining parts of the colon were stored as Swiss Rolls covered with Tissue-Tek, snap-frozen in liquid nitrogen and stored at –80 ˚C.

The flat-fixed colons of the animals sacrificed in week 7, 15 and 26 were stained with a 0.1% solution of methylene blue for 7 minutes to make ACF visible. They were examined at low magnification. The number of ACF was recorded and their size was determined by counting the number of crypts per ACF (AC/ACF).

From each group 7 large (≥ 5 mm) tumors were selected. From the selected tumors the half part attached to the colon was histologically processed, sectioned at 5 µm, and stained with haematoxylin and eosin. Serial sections were made whenever necessary to expose the stalk, if present. They were examined microscopically and the type of the tumors was established and recorded. Microscopic classification of the tumors was done according to the criteria described by Whiteley et al. [27]. The other halves of the large tumors mentioned above were used for isolation of RNA and real-time reverse transcription polymerase chain reaction (real-time RT-PCR). In the groups fed the diets with wheat bran and curcumin only few tumors larger than 5 mm had developed (2 and 3 respectively). Therefore, some smaller tumors were

also analyzed to attain the number of 7. Consequently, those additional small tumors were not available for microscopical classification.

RNA isolation

Colorectal tumors were disrupted using sonication and subsequent centrifugation over a QIAshredder spin column (QIAGEN, Westburg, Leusden, The Netherlands) in a buffer containing β-mercapthoethanol and guanidine thiocyanate (QIAGEN, Westburg, Leusden, The Netherlands). RNA was isolated from the supernatants using RNA-binding silica-gel membrane containing spin columns (QIAGEN, Westburg, Leusden, The Netherlands). In addition, RNA solutions were treated with RNase-free DNase I (QIAGEN, Westburg, Leusden, The Netherlands) to remove trace amounts of co-isolated DNA. RNA concentrations were determined using RiboGreen® RNA Quantitation Reagent (Molecular Probes, Leiden, The Netherlands).

cDNA synthesis and quantitative real-time RT-PCR

Total RNA (150 ng) was reversely transcribed using oligo(dT) VN_{15} primers (Promega, Madison, WI, USA) with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time RT-PCR were performed using TaqMan® probes (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) or QuantiTect™ SYBR® Green (QIAGEN, Westburg, Leusden, The Netherlands). The TaqMan® assays were performed in a total volume of 25 µl 1x TaqMan Universal Mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) in the iCycler iQ™ Real-Time PCR Detection System (Biorad, Veenendaal, The Netherlands). An initial denaturation step of 10 min at 95 ˚C was followed by 40-50 cycles of 95 ˚C for 15 s and 60 ˚C for 1 min. QuantiTect™ SYBR® Green PCR reactions were performed in a total volume of 20 µl 1x QuantiTect SYBR Green Master Mix (QIAGEN, Westburg, Leusden, The Netherlands) in the iCycler iQ™ Real-Time PCR Detection System. An initial denaturation step of 10 min at 95 ˚C was followed by 40-50 cycles of 95 ˚C for 15 s, 49 ˚C for 30 s and 72 ˚C for 20 s. Subsequently, a melting curve was generated by decreasing the setpoint temperature from 95 ˚C to 55 ˚C and measuring the fluorescence. Absolute amount of copies of the gene of interest in the experimental complementary DNA (cDNA) samples were calculated from the linear regression of a standard curve. The expression of the measured genes in each tumor was normalized for β-actin expression. Per tumor the expression of each gene was measured in duplicate. The sequences of the primers and the TaqMan® probes used for quantitative real-time RT-PCR were as follows: β-actin forward: 5'-TTC AAC ACC CCA GCC ATG T-3', reverse: 5'-GTG GTA CGA CCA GAG GCA TAC A-3', probe: 5'-CGT AGC CAT CCA GGC TGT GTT GTC C-3'. CA2 forward: 5'-AGG ACT TTG CAG TGC TGA AAG A-3', reverse: 5'-GCC CTG GCC ATC AGA TGA-3', probe: 5'-CCC TCA GTG GCT CCT ACA

GAT TGA TCC A-3'. CDK4 forward: 5'-AAG GAT CTG ATG CGC CAG TTT-3', reverse: 5'-CAG GTC CCG GTG AAC AAT G-3', probe: 5'-CGG CCT AGA TTT CCT TCA TGC A-3'. K-ras forward: 5'-AGG AAA CAA GTA GTA ATT GAT GGA GAA A-3', reverse: 5'-GTA CTG GTC CCT CAT TGC ACT GTA-3', probe: 5'-TCT CTT GGA TAT TCT CGA CAC AGC AGG TCA-3'. P53 forward: 5'-CCA TCA TCA CGC TGG AAG ACT-3', reverse: 5'-CCC AGG ACA GGC ACA AAC AC-3', probe: 5'-AAC CTC AAA GCT GTC CCG TCC CAG A-3'. TIMP-1 forward: 5'-GGG CTA CCA GAG CGA TCA CTT-3', reverse: 5'-AAG GTA TTG CCA GGT GCA CAA-3', probe: 5'-CCT GCC TGC CAC GGA ATC CAG A-3'. COX-2 forward: 5'-TCC CTT CGC CTC TTT CAA TG-3', reverse: 5'-GGA GGC ACT TGC GTT GAT G-3', probe: 5'-AAG ACC CGC AGG CTA CCA AGA CAG C-3'. CYP1A1 forward: 5'-ACA GAC CTC AGC TGC CCT ATC GT-3', reverse: 5'-TGA ATG GGA CAA AGG ATG AAT G-3', probe: 5'-AGG CCT TCA TCC TGG AGA CCT TCC G-3'. p27 forward: 5'-GCG ACC TGC GGC AGA A-3', reverse: 5'-GGG AAC CGT CTG AAA CAT TTT C-3' (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Statistical analysis

The multiplicity and size of ACF and colorectal tumors were analyzed using analysis of variance (ANOVA) followed by Student's *t*-test. Gene expression results were analyzed using a *t*-test. Tumor incidences were analyzed using Pearson's χ^2 test. A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.

Results

Survival of the animals

Survival of the animals was 90% in the control group, 100% in the wheat bran group, 94% in the curcumin group, 88% in the rutin group and 94% in the benzyl isothiocyanate group. Nine animals were found dead, 8 were euthanized because of poor health condition. The main cause of death and clinical problems was a tumor in the small intestines. One animal showed invagination of the colon due to a colon tumor. Other animals had haemorrhagic gastroenteritis, pleuropneumonia or had died of unknown cause. These animals were excluded from the study to ensure proper comparison of the different groups.

Food consumption, energy intake and terminal body weight

The different experimental groups had comparable food consumption: 13.9, 14.4, 13.5, 14.2 and 13.0 g/animal/day for the control, wheat bran, curcumin, rutin and benzyl isothiocyanate group, respectively. Since the different foods had similar energy content the overall mean energy intake of the animals was also comparable. Compared with the controls, the wheat bran group had the best growth performance, while the benzyl isothiocyanate group showed

slight growth retardation. This may be explained by the fact that, in contrast to some animals of other groups, none of the animals in the wheat bran group had showed health problems during the experiment.

Aberrant crypt foci (ACF)

All animals developed ACF. The ACF scores are presented in Table 6.1. ACF multiplicity at the different time points is also shown in Figure 6.1. In week 7, the highest numbers of ACF were present in the wheat bran and benzyl isothiocyanate groups. The lowest numbers were counted in the curcumin and rutin groups ($P < 0.05$, compared to the control group). The highest numbers of large ACF (4 or more AC/ACF) were present in the control, wheat bran and benzyl isothiocyanate groups.

Compared with week 7, the number of ACF was markedly decreased in week 15 in the control, wheat bran and benzyl isothiocyanate groups, but only slightly different in the curcumin and rutin groups. In general, in week 15, the numbers of ACF in all experimental groups were comparable, but the number of AC/ACF was higher in the wheat bran group than in the control group ($P < 0.05$). In week 26, the lowest number of ACF was found in the wheat bran group. The number of AC/ACF was highest in the rutin group ($P < 0.05$, compared to the control group). The other groups were comparable with the control group. At this time point the number of ACF was markedly decreased compared with week 7 in the control group, the wheat bran group and the benzyl isothiocyanate group, whereas it was increased in the curcumin group and rutin group (Figure 6.1). The mean number of AC/ACF increased from week 7 to week 26 in all groups.

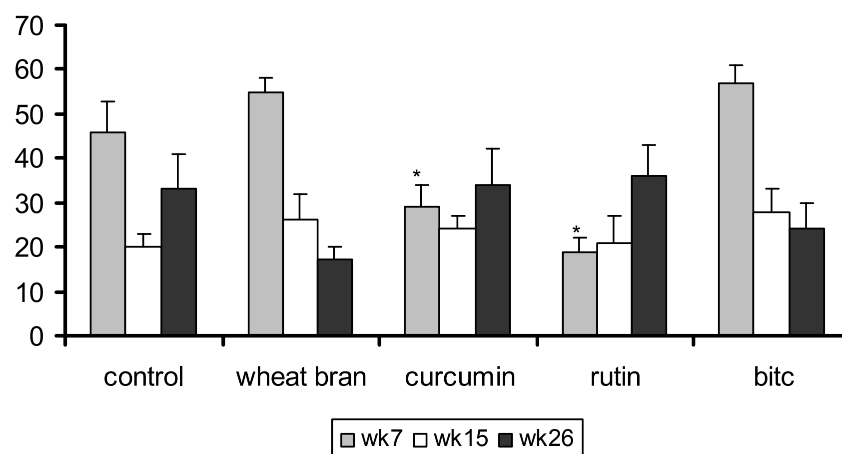

Figure 6.1. ACF multiplicity in rat colon after 7 weeks (grey bars), 15 weeks (white bars) and 26 weeks (black bars) in the control, wheat bran, curcumin, rutin and benzyl isothiocyanate (bitc) group. * $P < 0.05$, vs control group (Student's *t*-test).

Table 6.1. Overview of multiplicity of all ACF and of ACF with 4 or more aberrant crypts, number of aberrant crypts per ACF (mean ± SD), and of tumor incidence (%), multiplicity of tumors per animal (mean ± SEM), tumor size (mean ± SD) and tumor types used for RT-PCR.

	Experimental groups				
	Control	Wheat bran	Curcumin	Rutin	Benzyl isothiocyanate
	ACF after 7 weeks				
ACF multiplicity	46 ± 7	55 ± 3	29 ± 5 *	19 ± 3 *	57 ± 4
ACF ≥ 4 AC	21 ± 5	20 ± 1	11 ± 2	8 ± 2	21 ± 2
# AC / ACF	3.40 ± 0.53	3.14 ± 0.19	3.22 ± 0.38	3.16 ± 0.61	3.08 ± 0.08
	ACF after 15 weeks				
ACF multiplicity	20 ± 3	26 ± 6	24 ± 3	21 ± 6	28 ± 5
ACF ≥ 4 AC	13 ± 4	17 ± 4	16 ± 2	15 ± 5	18 ± 5
# AC / ACF	4.34 ± 0.26	4.76 ± 0.30 *	4.94 ± 1.62	4.78 ± 0.73	4.26 ± 1.07
	ACF and colorectal tumors after 26 weeks				
ACF multiplicity	33 ± 8	17 ± 3	34 ± 8	36 ± 7	24 ± 6
ACF ≥ 4 AC	25 ± 5	13 ± 2	27 ± 6	31 ± 5	18 ± 5
# AC / ACF	5.57 ± 0.73	4.97 ± 0.76	5.92 ± 0.90	6.83 ± 0.56 *	6.18 ± 0.92
Tumor incidence (%)	86	89	89	100	100
Tumor multiplicity	2.00 ± 0.36	1.12 ± 0.12	1.62 ± 0.50	1.86 ± 0.34	2.50 ± 0.54
Tumor size (mm)	2.3 ± 1.2	3.7 ± 2.6	3.8 ± 1.4 *	4.1 ± 2.9 *	3.2 ± 2.0
	Colorectal tumors after 8 months				
Number of animals	26	29	26	25	27
Tumor incidence (%)	92	76	100	88	96
Tumor multiplicity	3.29 ± 0.42	1.86 ± 0.23 *	2.27 ± 0.22 *	4.41 ± 0.44	3.85 ± 0.33
Tumor size (mm)	3.84 ± 0.44	2.95 ± 0.38	2.94 ± 0.26	4.31 ± 0.27	4.13 ± 0.30
Total number of tumors	79	41	59	97	100
	Tumor types used for RT-PCR				
Adenomas	2		1	1	
Carcinomas	5	2	2	6	7
Unknown[1]		5	4		
Total	7	7	7	7	7

AC: aberrant crypt; ACF: aberrant crypt focus.
* $P < 0.05$, vs control group (Student's *t*-test).
[1] No histologic diagnosis because completely used for RT-PCR.

Colorectal tumors

The incidence, multiplicity and size of colorectal tumors are presented in Table 6.1. The incidence is the percentage of animals bearing one or more colorectal tumors. The multiplicity is the number of colorectal tumors per tumor-bearing animal. The design of the study did not allow microscopical examination of all tumors to establish whether they were benign or malignant. Therefore, the table refers to the total number of macroscopically observed tumors. In the animals killed in week 7 no tumors were found. In week 15 again no tumors were found except for one small polyp in a rutin-fed animal. At necropsy in week 26, most animals had developed one or more colorectal tumors. The incidence was 86% in the control group, 89% in the wheat bran group, 89% in the curcumin group, 100% in the rutin group and 100% in the benzyl isothiocyanate group. The wheat bran group had the lowest multiplicity and the benzyl isothiocyanate group had the highest multiplicity, but the differences were not

statistically significant compared to the control group. The curcumin and rutin groups had the largest mean tumor size ($P < 0.05$, compared to the control group).

Upon final necropsy at 8 months, the highest incidence of colorectal tumors was found in curcumin-fed animals (100%); the wheat bran group showed the lowest incidence (76%). In the wheat bran and curcumin groups, the multiplicity of total tumors was significantly lower than in the control group ($P < 0.05$). The tumor multiplicity in the rutin and benzyl isothiocyanate groups was higher than that in the control group, but the difference was not statistically significant. The mean tumor size in all groups was not statistically significantly different from that in the control group. In all groups, the distribution of the tumors over the colon was comparable. Deep intramural processes, representing the typical macroscopic picture of a carcinoma, were generally slightly closer to the caecum than polypoid tumors.

Quantitative real-time RT-PCR

We investigated whether the effects of the different dietary compounds on the development of colorectal tumors was reflected by the expression of a subset of genes. Therefore, 7 tumors per group were analyzed for differential expression of 8 genes by quantitative real-time RT-PCR. Microscopic evaluation of these tumors showed that most tumors in the control, rutin and benzyl isothiocyanate group were carcinomas (Table 6.1). In the wheat bran and curcumin group 5 and 4 tumors, respectively, could not be microscopically classified, because of their small size. From the selected tumors total RNA was isolated of which cDNA was synthesized. For one tumor from the rutin group and two tumors from the benzyl isothiocyanate group no cDNA could be synthesized. Subsequently, expression of the following genes was analyzed: CA2, CDK4, COX-2, CYP1A1, K-ras, p27, p53 and TIMP-1. β-actin expression was measured in each tumor and used to normalize the expression of the genes of interest. Gene expression is presented in arbitrary units (Figure 6.2).

As can be seen in Figure 6.2A, quantitative real-time RT-PCR revealed that tumors from the wheat bran group had a significantly lower expression of CDK4, K-ras, p27, p53 and TIMP-1 as compared to tumors from the control group. Tumors from the curcumin group had significantly lower expression of COX-2 and K-ras as compared to tumors from the control group (Figure 6.2B). A significantly lower expression of COX-2 and a significantly higher expression of TIMP-1 were observed in tumors from the rutin group in comparison with tumors from the control group (Figure 6.2C).

In most tumors, expression of CYP1A1 was not detectable. Gene expression in tumors from the benzyl isothiocyanate group was not different from that in tumors from the control group (Figure 6.2D). However, all tumors from the benzyl isothiocyanate group were carcinomas. When carcinomas from the benzyl isothiocyanate group were compared specifically with the carcinomas from the control group, gene expression of K-ras was significantly lower (1.19 ± 0.08 vs 3.61 ± 0.79 in control group; $P < 0.05$).

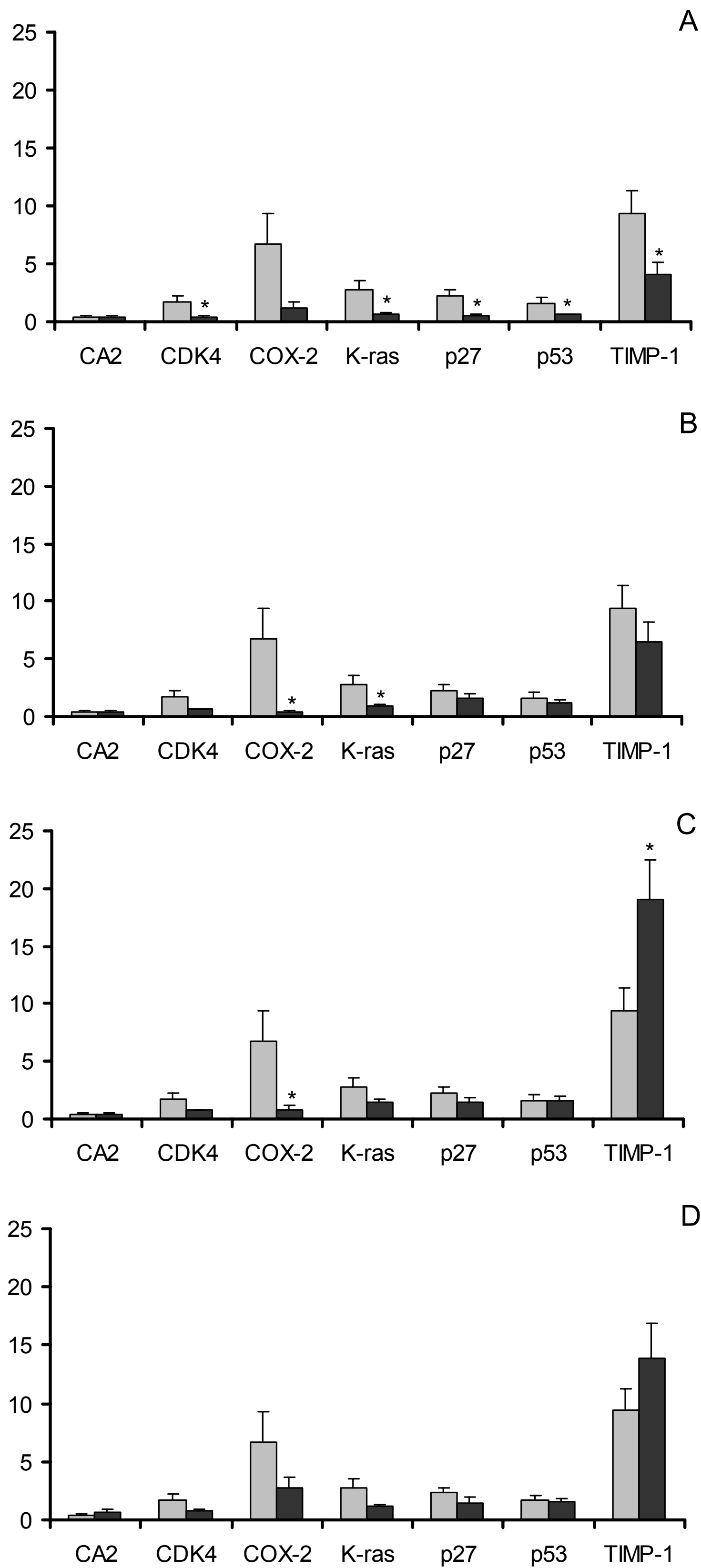

Figure 6.2. Expression of CA2, CDK4, COX-2, K-ras, p27, p53 and TIMP-1, corrected for β-actin expression, in control group (grey bars) and diet group (black bars), mean ± SEM. A: wheat bran, B: curcumin, C: rutin, D: benzyl isothiocyanate. *$P < 0.05$, compared to control group.

Best correlation between the gene expression in tumors and the tumor multiplicity in the diet groups was observed for TIMP-1 (correlation coefficient 0.97) and for p53 (correlation coefficient 0.84) (Table 6.2).

Putting together the gene expression and the tumor data demonstrates that the effects of the different dietary compounds on TIMP-1 expression correlated well with the effects of the dietary compounds on the ultimate tumor yield: TIMP-1 expression was lower in the wheat bran group than in the control group and it was higher in the rutin group than in the control group.

Table 6.2. Correlation of gene expression in tumors with tumor multiplicity and tumor incidence.

	Correlation coefficient	
Genes	Tumor multiplicity	Tumor incidence
CA2	0.238	0.262
CDK4	0.311	0.286
COX-2	0.201	0.110
K-ras	0.421	0.207
P27	0.468	0.664
P53	0.835	0.650
TIMP-1	0.974	0.228

Discussion

In the present study we investigated whether the effects of dietary compounds on the development of AOM-induced ACF and colorectal tumors correlated. In addition, the effects of these compounds on the expression of tumor-related genes were studied.

We assessed ACF by recording their total number and the number with 4 or more aberrant crypts, since it has been postulated that larger ACF may be more predictive than the total number of ACF [28, 29], and we assessed the size, expressed as number of AC/ACF. If the effect of a food compound on the formation of ACF at an early stage of carcinogenesis would have a reliable predictive value for either an increased or decreased risk of colorectal cancer, then at least one of the above mentioned features of ACF should consistently correlate with the development of colorectal tumors. It was concluded that this was not the case in the present study because of the following observations. In the wheat bran group, at 7 and 15 weeks, the ACF scores tended to be higher than in the control group and at 15 weeks the number of AC/ACF was higher ($P < 0.05$) than in the control group. However, at 8 months, the wheat bran group had a lower tumor multiplicity than the control group ($P < 0.05$). In comparison with the controls, the ACF multiplicity in the rutin group was lower at 7 weeks ($P < 0.05$), but at 26 weeks the number of AC/ACF and the tumor size were higher ($P < 0.05$), while at 8 months the tumor multiplicity and size were highest of all groups. Another notable finding was that although the ACF figures in the wheat bran and benzyl isothiocyanate groups

were very similar at 7 and 15 weeks, the numbers of tumors found in the benzyl isothiocyanate group at 26 weeks and at 8 months were more than twice as high as those found in the wheat bran group. In the curcumin group both the ACF multiplicity at 7 weeks and the tumor multiplicity at 8 months were lower than those in the control group ($P < 0.05$). Summarizing, a good prediction of ACF for colorectal tumors was only observed for the curcumin group at 7 weeks. In all other groups, ACF had no predictive value.

Failure of ACF to predict the development of colorectal tumors is consistent with results of previous studies [30-34]. In contrast, in other occasions there was a correlation between occurrence of ACF and colorectal tumors [4, 35-38]. These conflicting results indicate that recording number and size of ACF alone cannot be used as a reliable screening assay. Nevertheless, ACF are putative preneoplastic lesions and studying them may yield relevant knowledge about the carcinogenic process. It is interesting to note that the experimental diets apparently had a different effect on their development. In rats and mice ACF appear in the colon and rectum about 2 weeks after treatment with a carcinogen. In the following months their number increases and they become larger. Next, some ACF may disappear because they regress, most will stay present, probably as innocent bystander lesions, while one or more of these persistent ACF may develop into a tumor [39]. This general pattern is applicable to the wheat bran and benzyl isothiocyanate groups in the present study. In the curcumin and rutin groups, however, the number of ACF continued to increase, especially the larger ones. The controls showed an increased number after an initial decrease. This means that a dietary compound may influence not only the number of ACF, but it also may determine i) the moment they appear, ii) the moment they start to regress or become dysplastic and iii) their growth rate. Since it is unpredictable how a potential chemoprotective agent may influence these factors, the ACF score established at an arbitrary time point is unreliable as predictive factor for inhibition of colorectal cancer.

In the present study, wheat bran effectively inhibited colorectal carcinogenesis, which confirmed the observations of numerous other investigators [4, 38, 40-46]. Furthermore, Alberts et al. [47] found that a dietary supplement of 13.5 g wheat bran per day inhibited DNA synthesis and rectal mucosa cell proliferation in high-risk patients. Although this was only a pilot study, the results suggested that relatively small amounts of dietary fiber may already have a preventive effect. The protective effect of wheat bran against colorectal cancer is associated with stool bulking, a shorter intestinal transit time and the formation of short chain fatty acids, of which butyrate is probably the most important one [46, 48].

Seven out of 41 tumors in the wheat bran group differed significantly from an equal number of tumors in the control group in expression of CDK4, K-ras, p27, p53 and TIMP-1. Down-regulation of protein levels of p53 was also found in invasive colonocytes treated with short chain fatty acids [49]. In epidermal cells co-expression of ras and CDK4 promoted cell growth and stimulated development of invasive neoplasia [50]. Butyrate down-regulated gene

expression of N-ras in colon carcinoma cells in vitro [51]. Reddy et al. [52] demonstrated that specifically the lipid fraction of wheat bran could down-regulate COX-2 protein expression. In this study, however, gene expression of COX-2 in colorectal tumors was not significantly down-regulated by wheat bran. Protein levels of p27 are low in colorectal cancers, and expression of p27 in tumors is reported to be correlated to survival rate [53]. Tumors in the wheat bran group had a lower expression of p27 than tumors in the control group. In contrast to the consistent effect of wheat bran on gene expression of CDK4, K-ras, p53 and TIMP-1, the effect of wheat bran on gene expression of p27 does not seem to correspond with the inhibitory effect of wheat bran on colorectal cancer.

Although in the present study after 8 months dietary curcumin had a protective effect on tumor multiplicity only, it has been shown to inhibit both the incidence and multiplicity of colorectal tumors in animal models [5, 54, 55]. The protective effect of curcumin has been related to its antioxidant and antimutagenic effects [56], to its influence on arachidonic acid metabolism [5], and to its ability to inhibit prostaglandin synthesis [57] and to enhance apoptosis [58]. Tumors of the curcumin group showed down-regulated gene expression of COX-2 and K-ras in comparison with tumors of the control group. These results were in accordance with published studies, in which tumors were induced by other carcinogens. Rao et al. [5] reported lower COX-2 activity in tumors induced by AOM from rats that were fed a diet with 2000 ppm curcumin. In mice curcumin decreased expression of ras oncogenes in carcinogen-induced skin tumors [59]. Further, curcumin decreased expression of ras oncogenes induced by diethylnitrosamine in rat liver [58] and lowered COX-2 protein and mRNA levels in human colon cancer cells *in vitro* [60].

At 26 weeks, the tumor incidence and tumor size of the rutin group was enhanced compared to the control group. After 8 months, the total number of tumors was higher than in to the control group, but the incidence was comparable and no significant differences with respect to tumor multiplicity and size were observed in the rutin group. Several studies did not reveal a carcinogenic or tumor-promoting effect of rutin or quercetin [2, 61-63]. Furthermore, quercetin has been shown to exert chemoprotective properties such as antioxidant activity [12] and the ability to inhibit arachidonic acid metabolism [64]. On the other hand, Pereira et al. [55] found enhancement of colorectal tumorigenesis in AOM-treated rats. The lack of protection against carcinogenesis in this study could not be explained.

Even though rutin did not protect against the development of colorectal cancer, gene expression of COX-2 was decreased in tumors of the rutin group, compared to tumors of the control group. In AOM-treated rats fed rutin for 7 days, p27 levels in colorectal tumors did not change [24]. In this study rats were fed rutin for a longer period and also no effect on expression of p27 in colorectal tumors was found. In the rutin group the expression of TIMP-1 in colorectal tumors was increased compared to the control group. In contrast, in humans supplemented with quercetin for 14 days plasma levels of TIMP-1, mRNA and protein were

decreased [65]. It is known that high TIMP-1 expression is correlated with tumor progression and liver metastasis [26, 66]. TIMP-1 may exert its effect on colorectal carcinogenesis through stimulation of cell growth, which may be influenced by interaction with ras [67, 68]. Interestingly, in this study TIMP-1 gene expression was down-regulated in tumors of rats fed a wheat bran diet, which protected against development of colorectal tumors, and up-regulated in tumors of rats fed a rutin diet, which showed a trend to enhance colorectal tumor development. Thus, increased and decreased TIMP-1 gene expressions in colorectal tumors seem to correlate with promotion and inhibition of colorectal cancer, respectively. It is clear that this result should be interpreted with caution because only a limited number of tumors was available for analysis. Furthermore, TIMP-1 is involved in tumor metastasis and from the tumors analyzed carcinomas were prevalent. Further studies are needed to verify the role of TIMP-1 expression in colorectal carcinogenesis.

In contrast with the results of the present study, Sugie et al. [3] found that rats fed a diet containing 0.04% benzyl isothiocyanate showed decreased development of colorectal tumors. This may be explained by the difference in study protocol. Sugie et al. [3] used methylazoxymethanol-treated female ACI/N rats and the incidence and multiplicity of colorectal tumors was reduced if benzyl isothiocyanate was given during initiation, but not if benzyl isothiocyanate was given during promotion. Although benzyl isothiocyanate did not protect against development of colorectal cancer in this study, carcinomas from the benzyl isothiocyanate group had a lower expression of K-ras than carcinomas from the control group.

In summary, it can be concluded that wheat bran and curcumin had a protective effect on the development of colorectal tumors. Despite their assumed protective properties, the rutin and benzyl isothiocyanate diets showed an enhancing (although not statistically significantly) rather than inhibitory effect on colorectal carcinogenesis. It should be noted that the study design did not allow a complete analysis of the tumor samples: several small tumors were not available for histopathological analysis because they were used for RNA analysis. Therefore, it was not possible to detect effects such as a shift from adenomas to carcinomas or vice versa. Consequently, the occurrence of such effects cannot be excluded.

The number and size of ACF were not considered to be suitable as biomarker for colorectal cancer. Recent investigations indicate that other characteristics of ACF, such as beta-catenin or mucin content, may be more predictive of colorectal carcinogenesis [69, 70].

The gene expression results clearly imply a correlation between the expression of TIMP-1 and colorectal carcinogenesis. At this stage it would be incorrect to suggest that TIMP-1 expression is predictive for the development of colorectal tumors, since the tumors were already formed. The possible role of TIMP-1 in development of colorectal cancer has to be verified in further studies, e.g. by measuring expression of TIMP-1 and related genes like matrix metalloproteinases (MMPs) in ACF and normal colon tissue.

ACF did not predict development of colorectal tumors in the different diet groups. Potential biomarker genes for colon cancer prevention were evaluated by gene expression measurement in tumors from the different diet groups. Next, this could be extended towards gene expression measurements in ACF and normal intestinal tissue. In addition, by using microarrays for gene expression profiling, more potential new biomarkers for colorectal cancer can be found [71]. Next, we will study gene expression changes in intestinal cells of AOM-treated rats in response to different food compounds.

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Gene expression profiling of effects of plant compounds in relation to colon carcinogenesis

SUBMITTED FOR PUBLICATION

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Abstract

Several plant compounds can exert beneficial effects on intestinal health, e.g. they can act as antioxidants and protect against oxidative stress and intestinal inflammation. In addition, these compounds can possibly protect against development of colon cancer. In this study, effects of four plant compounds on small intestinal gene expression profiles were studied and related to colon tumor development.

Azoxymethane-treated rats were fed a control diet or a diet with wheat bran (10%), curcumin (0.2%), rutin (4%) or benzyl isothiocyanate (0.04%) for 8 months. Colon tumor development was studied at several time points. Gene expression profiles were measured in small intestinal scrapings after 7 and 26 weeks, using a cDNA microarray with 3000 rat genes. At both time points, the expression profile of a subset of significantly differentially expressed genes was predictive of colon tumor multiplicity at the end of the study, as demonstrated by multivariate statistical analysis. Functional classes of genes that contributed to the predictive value of gene expression profiles at week 7 included cell proliferation genes, ribosomal genes and oxidoreductase genes. All compounds affected expression of immune-related genes. Other differentially expressed genes that could play a role in colon cancer development include proteasome genes, metallothionein and urokinase plasminogen activator.

This study points out that gene expression profiling of intestinal tissue could be useful in the search for biomarkers for effects of food compounds on intestinal cancer development and promotion of intestinal health, especially focusing on the relationship between nutrient-gene interaction in the small and large intestines.

Introduction

Food compounds can exert a range of effects throughout the gastrointestinal tract, mainly related to digestion, metabolism and uptake of nutrients. At the same time food compounds can exert beneficial effects in relation to intestinal health. For example, many plant compounds can act as an antioxidant, thereby preventing mucosal damage from reactive oxygen species and inflammation. Chronic inflammation of intestinal tissue can lead to diseases like inflammatory bowel disease [1, 2]. Furthermore, diet and dietary factors are thought to be related to development of intestinal cancer, especially colon cancer. It has been estimated that as much as 50 percent of colon cancer can be avoided by dietary changes [3]. Many studies reported a protective effect of fruits and (specifically raw and green) vegetables on colon cancer development [4, 5] and various plant compounds probably contribute to this effect. A range of mechanisms of prevention of intestinal cancer by plant compounds have been described, including effects on cell growth and apoptosis, detoxification of carcinogens, induction of antioxidant enzymes in intestinal tissue or anti-inflammatory action [6, 7].

An 8-month study was conducted to study the anti-carcinogenic effects of four different plant compounds in azoxymethane-treated rats. The compounds studied were wheat bran (10%), curcumin (0.2%), rutin (4%) and benzyl isothiocyanate (BITC) (0.04%). Previous studies pointed out that these compounds could be promising with regard to cancer prevention. Wheat bran, the grind husk of wheat, protected against the development of aberrant crypt foci (ACF) and tumors in colon of rats treated with a carcinogen [8, 9]. This protective effect is often related to conversion of the fiber in wheat bran into short chain fatty acids by colonic microflora. However, wheat bran also contains other components, like phytic acid and phenolic compounds, which can contribute to its health-promoting effect [10]. Curcumin is used as a spice and coloring agent and can act as an anti-inflammatory agent and an antioxidant [11, 12]. In rats and mice curcumin inhibited development of chemically induced ACF and colon tumors [13-15]. Flavonoids occur in plants mainly as glycosides. Rutin is a glycoside conjugate of quercetin. Both rutin and quercetin can act as antioxidants. Contrary observations have been reported with respect to rutin and quercetin. Some studies report that rutin and/or quercetin protected against development of colon tumors [16], while other studies found no effect of quercetin or even an increase in the number of tumors [14, 17]. Isothiocyanates are present in cruciferous vegetables as glucosinolates and can act as antioxidants [18]. Benzyl isothiocyanate has been found to protect against development of colon cancer in carcinogen-treated rats [19].

When development of aberrant crypt foci (ACF) at three intermediate time points (week 7, 15 and 26) was compared to tumor development after 8 months, it was concluded that ACF multiplicity at any of the time points did not predict development of colon tumors after 8 months [20]. Large-scale expression profiling with microarrays might be very useful in search for new biomarkers of colon tumor development. Therefore, expression changes of 3000

genes were measured in small intestinal epithelial scrapings using microarrays (1) to investigate whether gene expression profiles in small intestine correlate to and predict colon cancer development and if so, which functional groups of genes are involved and (2) additionally to study differential expression of genes in response to wheat bran, curcumin, rutin and benzyl isothiocyanate in small intestinal tissue and to put the (groups of) differentially expressed genes in context of possible effects of the compounds on gastrointestinal health and diseases, e.g. intestinal cancer and inflammation.

Materials and Methods

Animals and diets

Two hundred and fourty male specific-pathogen-free Fischer 344 rats (Charles River Deutschland, Sulzfeld, Germany), four weeks old, were divided into 5 groups of 48 animals each. The control group was fed an AIN^{93}-based diet. The other four groups were fed the same diet supplemented with either 10% (w/w) wheat bran (Meneba Feed Ingredients, Rotterdam, The Netherlands), 0.2% (w/w) curcumin (Fisher Scientific BV, 's-Hertogenbosch, The Netherlands), 4% (w/w) rutin (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 0.04% (w/w) benzyl isothiocyanate (Sigma Aldrich, Zwijndrecht, The Netherlands). 4% rutin is approximately equimolecular to 2% quercetin [16]. The supplements were added to the diets at the expense of wheat starch.

Treatment and housing

All animals were treated with weekly subcutaneous injections with AOM (Sigma-Aldrich, Zwijndrecht, The Netherlands), 15 mg/kg body weight, for 3 weeks. The first injection was given one week after the start of the experiment. The animals were housed in macrolon cages with bedding, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10 per hour. Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 hours light, 12 hours dark.

Necropsy and tissue collection

Seven and 26 weeks after the start of the experiment 6 to 9 animals per group were killed by decapitation under O_2/CO_2 anesthesia. The small intestine was divided in 4 pieces of equal length. From each of these pieces mucosa was scraped and stored in RNA-later (Ambion, Austin, Texas, USA) at –80 °C, to prevent breakdown of RNA. In addition, at week 7, 15 and 26 colon tissue was collected for counting ACF and at week 26 and at 8 months colon tumors were collected [20].

RNA isolation

Intestinal scrapings of the last quarter of the small intestine (closest to colon) were used for RNA isolation. After removal of RNA later, the scrapings were homogenized in Trizol (Life Technologies S.A., Merelbeke, Belgium) and total RNA was isolated according to the manufacturer's protocol. RNA clean-up and Dnase digestion was performed using the RNeasy mini kit (Qiagen, Hilden, Germany). RNA quantity was determined spectrophotometrically. RNA was checked for purity and stability by gel electrophoresis. RNA yield and quality of the scrapings from the rutin group in week 7 was too low, so this group could not be included in gene expression analysis.

Transcriptomics experimental design

Per diet group (control, wheat bran, curcumin, rutin, benzyl isothiocyanate) RNA from 3 to 7 animals was pooled. These RNA samples were hybridized to the microarray together with a reference sample. This reference sample consisted of pooled RNA from control group animals from the different time points (9 animals in total). RNA from the control group and from each of the four diet groups was labeled with Cy5 and the reference RNA was labeled with Cy3. Hybridizations were performed in duplicate.

cDNA microarray preparation, labeling and hybridization

In this study cDNA microarrays were used that contained about 3000 different sequence-verified cDNA clones. These arrays were constructed as described previously [21], with the only difference that each cDNA clone was spotted twice on the microarray.

Total RNA (25 µg) was labeled using CyScribe first-strand cDNA labeling kit (Amersham Biosciences, Freiburg, Germany). During reverse transcription of the RNA, Cy3- or Cy5-labelled dUTP was built into the cDNA. After incubating the reverse transcription reaction mixture for 90 minutes at 42 ºC, RNA was hydrolyzed by adding NaOH. After neutralization, free nucleotides were removed from the solution using AutoSeq G50 columns (Amersham Biosciences, Freiburg, Germany).

The amount of cDNA obtained and the incorporation rate of the fluorophore were determined spectrophotometrically. Prior to hybridization, labeled cDNAs of both sample and reference were mixed and dissolved in 30 µl EasyHyb hybridization buffer (Roche Diagnostics, Mannheim, Germany). Yeast tRNA (100 µg, Life Technologies S.A., Merelbeke, Belgium) and Poly dAdT (20 µg, Amersham Biosciences, Freiburg, Germany) were added to avoid non-specific binding. The hybridization mixture was denatured for 1.5 minutes at 100 °C and pipetted onto a pre-hybridized microarray slide which was covered with a plastic coverslip and, embedded in a slide incubation chamber (Corning, Life Sciences, Schiphol, The Netherlands), submerged in a water bath for at least 16 hours at 42°C. After hybridization, slides were washed by firm shaking in 0.5x SSC buffer in a 50 ml tube and two times 10

minutes in 0.2x SSC on a mechanical shaking platform. Slides were dried quickly by centrifugation at 700 rpm.
Slides were scanned with a ScanArray Express confocal laser scanner (Perkin Elmer Life Sciences, USA) and Imagene 4.2 (Biodiscovery Inc., Los Angeles, USA) was used to extract data from the images, with automatic flagging of weak or negative signals and spots with non-homogeneous signal.

Data analysis
The multiplicity of colorectal tumors was analyzed using analysis of variance (ANOVA) followed by Student's t-test. A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.
Microarray data were imported into SAS Enterprise guide V2 (SAS Institute Inc., Cary, USA). Spots with a signal/background ratio less than 2 or spots that were flagged by the Imagene software were not included in the data analysis. For each spot, local background intensity was subtracted from mean signal intensity and the expression ratio was calculated by dividing background-corrected signal intensity of the sample by the background-corrected signal intensity of the reference. Expression ratios were then log transformed (base 2) and normalized per slide using an intensity-dependent method (Lowess) [22]. Data were transferred to Microsoft Excel 2000 (Microsoft Corporation, USA) for further analysis.
Since each array contained two spots of each clone and each sample was analyzed twice, four expression ratios were calculated for each gene. Only clones with maximally one missing value were included in the data analysis.
The multivariate statistical method 'partial least squares' was used to investigate to what extent expression profiles of genes in small intestinal tissue were predictive of colon tumor development. In order to minimize inclusion of false positives in this analysis, a subgroup of differentially expressed genes was identified by ANOVA ($P<0.05$), both at week 7 and at week 26. Subsequently, these subgroups of differentially expressed genes at week 7 and at week 26 were used in the multivariate statistical analysis. By using this approach, small intestinal expression profiles at week 7 and week 26 were correlated to tumor multiplicity in the colon after 8 months.
In addition to the multivariate statistical analysis, SAM (significance analysis of microarrays) [23] was used to assess significance of differences in expression between each diet group and the control group (AOM treatment only). Genes with a q-value (threshold for chance of false positive detection for significant genes) less than 10% were selected as significantly differentially expressed. For all genes average expression ratios were used to calculate ratios of expression in each diet group compared to the control group.

Genbank accession numbers were used to find gene name and information on function of the genes in the NCBI databases (Locuslink, Unigene: http://www.ncbi.nlm.nih.gov/) and in the SOURCE database (http://genome-www5.stanford.edu/cgi-bin/source/sourceSearch).

Results

After 8 months no significant differences in tumor incidence were found between the control group and the four diet groups. Colon tumor multiplicity (number of tumors per tumor-bearing animal) was significantly lower in the wheat bran and the curcumin group, compared to the control group (figure 7.1).

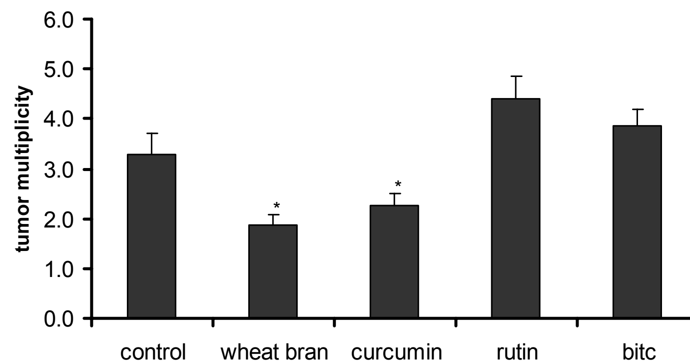

Figure 7.1. Tumor multiplicity (number of tumors per tumor-bearing animal) after 8 months in AOM-treated rats fed a control diet or a diet with wheat bran, curcumin, rutin or benzyl isothiocyanate (bitc) for 8 months. *: $P<0.05$ compared to control group.

Expression profiles in small intestine at week 7 and week 26 were correlated to colon tumor multiplicity after 8 months by using the multivariate statistical method partial least squares (PLS). By using this approach it was investigated to what extent these expression profiles predicted colon tumor development. Only genes with significant differential expression across the 5 groups (control diet, wheat bran, curcumin, rutin, benzyl isothiocyanate) were included in the analysis. Both at week 7 and at week 26, this subgroup of differentially expressed genes consisted of about 400 genes.

A high correlation was observed between colon tumor multiplicity measured after 8 months and colon tumor multiplicity predicted by expression profiles of the subsets of differentially expressed genes at week 7 and at week 26 ($R^2>0.98$) (figure 7.2). This could indicate that small intestinal gene expression profiles at week 7 and week 26 might be predictive of colon tumor development after 8 months. If gene expression profiles might be useful as early biomarkers, it is most interesting to focus on the data obtained at the early time point, week 7.

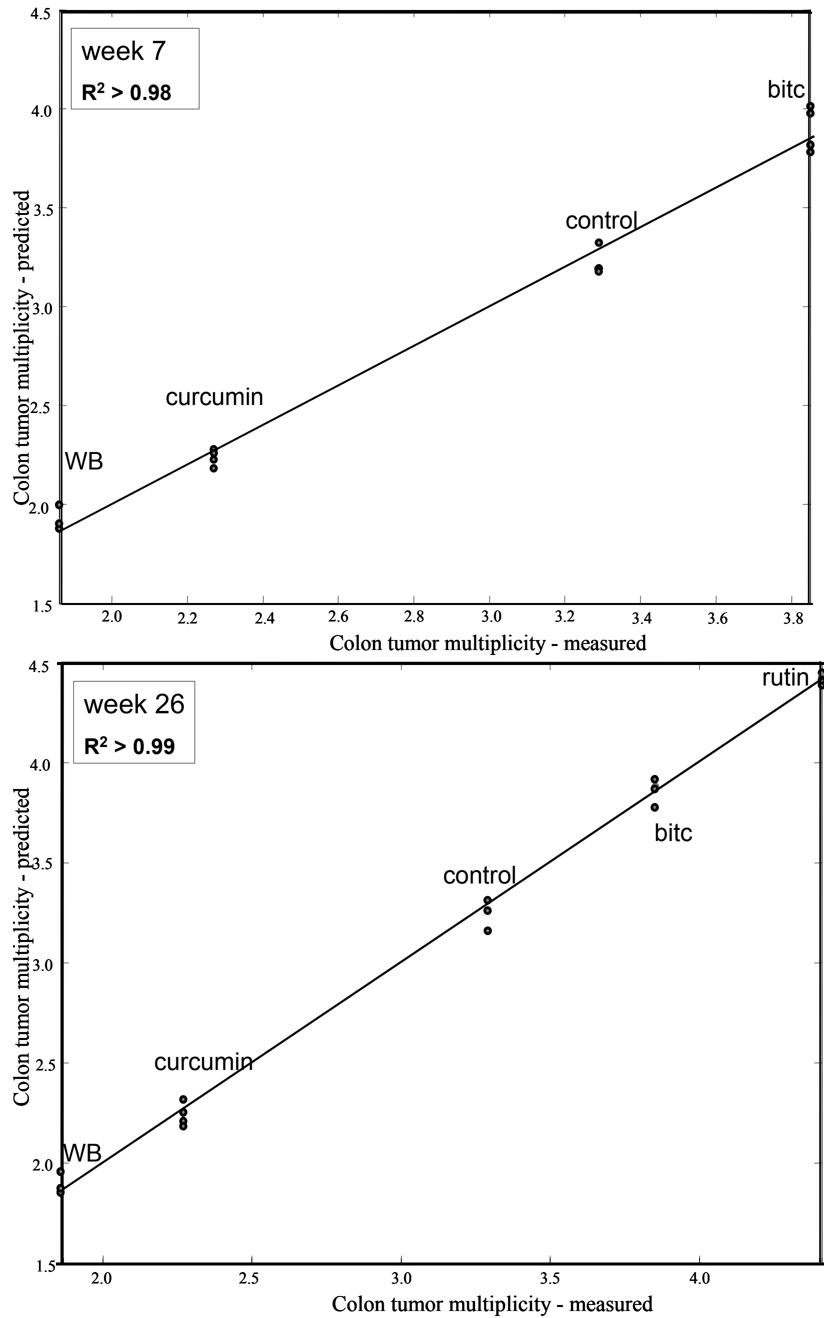

Figure 7.2. Correlation between colon tumor multiplicity measured and colon tumor multiplicity predicted by gene expression profiles of genes that are differentially expressed in small intestinal epithelium at week 7 (upper) or predicted by gene expression profiles of genes that are differentially expressed in small intestinal epithelium at week 26 (lower). The multivariate statistical analysis method partial least squares was used to investigate possible prediction of colon tumor multiplicity after 8 months by gene expression profiles in small intestine at week 7 or week 26. WB: wheat bran, bitc: benzyl isothiocyanate.

Next, genes whose expression profile contributed mostly to the correlation with tumor multiplicity after 8 months were selected from the subgroup of differentially expressed genes at week 7. These genes were grouped according to function and are listed in table 7.1. A positive correlation indicates that a higher expression of the gene coincides with a higher colon tumor multiplicity as well as a lower expression of the gene coincides with a lower colon tumor multiplicity. A negative correlation indicates that a higher expression of the gene coincides with a lower colon tumor multiplicity as well as a lower expression of the gene coincides with a higher colon tumor multiplicity. For example, in table 7.1 it can be seen that a number of genes involved in cell proliferation, cell death and cytoskeleton were negatively correlated to colon tumor multiplicity. Furthermore, the majority of the genes related to oxidative stress or oxidoreductase activity were positively correlated to tumor multiplicity (table 7.1). Also, a few ribosomal genes were positively correlated. Other groups of genes that contributed to the correlation were genes involved in transcription regulation, genes encoding for proteins with protein kinase activity and genes involved in signal transduction (table 7.1).

Table 7.1. Functional groups of genes with significant differential expression among diet groups at week 7 that correlate (positively or negatively) with tumor multiplicity after 8 months

Accession Number	+/-[a]	Gene Name	Gene Symbol	Function
AA818413	-	clusterin	Clu	cell death
AA819611	-	insulin-like growth factor binding protein 3	Igfbp3	cell growth
AA859566	-	upregulated by 1,25-dihydroxyvitamin D-3	Vdup1	cell proliferation
AA874917	-	biglycan	Bgn	cytoskeleton
AA965256	-	fast myosin alkali light chain	Mlc3	cytoskeleton
AA875581	-	myosin regulatory light chain	Mrlcb	cytoskeleton
AA818522	+	similar to microsomal glutathione S-transferase 3 (LOC289197), mRNA	-	drug metabolism
AA964981	+	cytochrome P450, subfamily IVF, polypeptide 14 (leukotriene B4 omega hydroxylase	Cyp4f14	drug metabolism
AI136404	+	cytochrome P450 3A9	Cyp3a9	drug metabolism
AA963724	-	cytochrome P450, subfamily 2F, polypeptide 1	Cyp2f1	drug metabolism
AA964989	+	Protein disulfide isomerase (Prolyl 4-hydroxylase, beta polypeptide)	P4hb	electron transport
AA924540	+	xanthine dehydrogenase	Xdh	electron transport, oxidoreductase activity
AA899832	+	cytochrome c oxidase, subunit VIa, polypeptide 1	Cox6a1	electron transport, oxidoreductase activity
AA955550	-	cytochrome c oxidase subunit Vb	Cox5b	electron transport, oxidoreductase activity
AA859399	+	Metallothionein	Mt1a	oxidative stress, metal ion binding
AA900218	+	Metallothionein	Mt1a	oxidative stress, metal ion binding
AA899815	+	phytanoyl-CoA hydroxylase (Refsum disease)	Phyh	oxidoreductase activity
AI045289	+	similar to NADH dehydrogenase (ubiquinone) Fe-S	-	oxidoreductase activity

Accession Number	+/-[a]	Gene Name	Gene Symbol	Function
		protein 2 (LOC289218), mRNA		
AI072330	+	lactate dehydrogenase A	Ldha	oxidoreductase activity, glycolysis
AA996424	-	lysyl oxidase	Lox	oxidoreductase activity
AI029934	+	homeo box, msh-like 1	Msx1	transcription regulation
AA819147	+	amino-terminal enhancer of split	Aes	transcription regulation
AA996525	-	POU domain, class 3, transcription factor 4	Pou3f4	transcription regulation
AA859478	-	Aryl hydrocarbon receptor	Ahr	transcription regulation
AA875354	+	ribosomal protein S15a	Rps15a	protein biosynthesis
AA859635	+	similar to large subunit ribosomal protein L36a (LOC292964), mRNA	-	protein biosynthesis?
AA818640	+	similar to ribosomal protein S18, cytosolic [validated] - rat (LOC313379), mRNA	-	protein biosynthesis?
AA924704	+	proteasome (prosome, macropain) subunit, beta type 1	Psmb1	protein catabolism (ubiquitin-dependent)
AI044968	+	mast cell protease 9	Mcpt9	proteolysis
AI060106	-	hepsin	Hpn	proteolysis
AI058790	+	LIM motif-containing protein kinase 2	Limk2	protein kinase activity
AA900032	-	S6 kinase	Rps6kb 1	protein kinase activity
AA997367	-	p21 (CDKN1A)-activated kinase 3	Pak3	protein kinase activity
AI071795	-	casein kinase 1, delta	Csnk1d	protein kinase activity
AI059997	+	stress activated protein kinase alpha II	Mapk9	signal transduction
AA817697	+	ADP-ribosylation-like 2	Arl2	signal transduction
AA957483	-	transmembrane 4 superfamily member 3	Tm4sf3	signal transduction

[a]: + indicates a positive correlation, meaning that a higher expression of the gene coincides with a higher colon tumor multiplicity as well as a lower expression of the gene coincides with a lower colon tumor multiplicity. – indicates a negative correlation meaning that a higher expression of the gene coincides with a lower colon tumor multiplicity as well as a lower expression of the gene coincides with a higher colon tumor multiplicity.

Table 7.2. Number of genes significantly differentially expressed in small intestine in the four different diet groups, compared to the control group, at week 7 and week 26.

	Differentially expressed genes	
	Week 7	Week 26
Wheat bran	55 up, 7 down	0
Curcumin	6 up, 7 down	0
Rutin	NA*	28 up
Benzyl isothiocyanate	74 up, 7 down	29 up, 18 down

* Effects of rutin at week 7 were not analyzed.

In addition to the multivariate statistical analysis described above, which simultaneously analyzed the complete dataset of all five groups, the gene expression profile of each individual diet group was compared to the gene expression profile in the control group. Table 7.2 shows the number of statistically differentially expressed genes in each diet group compared to the control group at week 7 and 26. No significant differences in gene expression were found in the wheat bran and the curcumin group, compared to the control group, at week 26 (Table 7.2). At both time points the largest number of differentially expressed genes was found in the benzyl isothiocyanate group. In general, the induction or repression of gene expression of significantly differentially expressed genes ranged from 1.2 to 2.4-fold.

A number of genes were expressed at a significantly higher or lower level in more than one diet group. Expression of a gene similar to ilvB (bacterial acetolactate synthase)-like isoform 1 was upregulated by wheat bran, curcumin and benzyl isothiocyanate at week 7 (figure 7.3). Expression of this gene was significantly downregulated by benzyl isothiocyanate at week 26.

Both in benzyl isothiocyanate and curcumin diet groups expression of metallothionein (Mt1a) was induced at week 7 (figure 7.4). Phospholipase A2 group IIa (Pla2g2a) showed an opposite response in the wheat bran (upregulation) and the curcumin group (downregulation) (figure 7.5). At week 7 20 genes were significantly differentially expressed in both the wheat bran and the benzyl isothiocyanate group. In this group, 17 genes are expressed at a higher level and three genes are expressed at a lower level in small intestinal scrapings of both the wheat bran group and the benzyl isothiocyanate group, compared to the control group (Table 7.3). Genes in this subgroup were involved in several processes including metabolism, immune-related processes and proteolysis. Significant expression changes of the genes ranged from 1.2- to 1.8-fold.

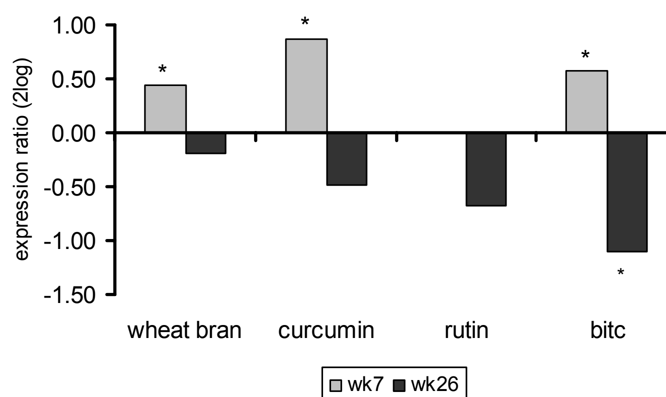

Figure 7.3. Expression changes of 'a gene similar to ilvB (bacterial acetolactate synthase)-like isoform 1' in small intestinal scrapings from wheat bran, curcumin, rutin and benzyl isothiocyanate (bitc) diet group at week 7 (grey bars) or week 26 (black bars). Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). * indicates significant difference in expression between diet group and control group.

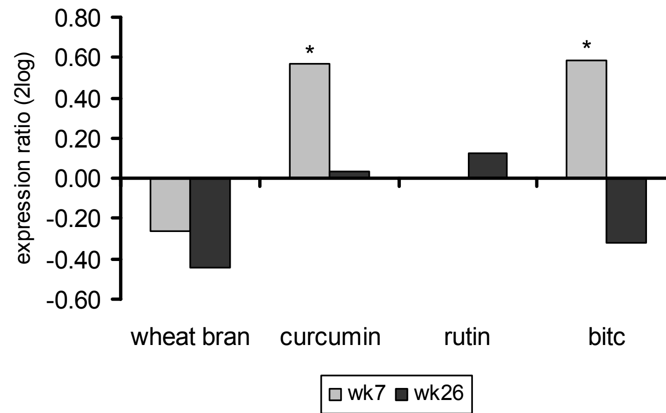

Figure 7.4. Expression changes of metallothionein in small intestinal scrapings from wheat bran, curcumin, rutin and benzyl isothiocyanate (bitc) diet group at week 7 (grey bars) or week 26 (black bars). Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). * indicates significant difference in expression between diet group and control group.

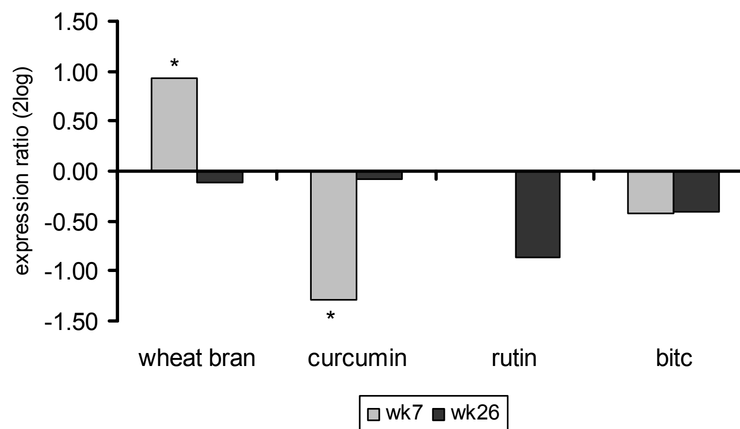

Figure 7.5. Expression changes of phospholipase A2 group IIa in small intestinal scrapings from wheat bran, curcumin, rutin and benzyl isothiocyanate (bitc) diet group at week 7 (grey bars) or week 26 (black bars). Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). * indicates significant difference in expression between diet group and control group.

Only a limited number of genes were differentially expressed in intestinal mucosa from the curcumin group, exclusively at week 7. In addition to differential expression of the genes mentioned above (MT1a, Pla2g2a and a gene similar to bacterial acetolactate synthase homolog) expression of ‘Rat Ig active kappa-chain mRNA VJC-region from immunocytoma IR2’, a gene with strong similarity to beta actin, a gene similar to alpha-1 catenin and a gene similar to RAKb were downregulated in small intestinal scrapings in the curcumin group.

Table 7.3. Genes significantly higher or lower expressed in both wheat bran and benzyl isothiocyanate groups compared to control group at week 7.

Accession number	Gene Name	Function
Significant higher expression in wheat bran and benzyl isothiocyanate group compared to control group		
AA859488	calreticulin	Calcium ion binding
AA998869	protein tyrosine phosphatase, non-receptor type substrate 1	Cytoskeleton, cell adhesion, protein binding
AA859605	intercellular adhesion molecule 1	Cell adhesion, defense response
AA997294	T-cell receptor gamma chain	Immune-related, cell growth?
AI043642	mal, T-cell differentiation protein 2	Immune-related?
AA963724	cytochrome P450, subfamily 2F, polypeptide 1	Drug metabolism
AI136203	fructose-1,6- biphosphatase 1	Metabolism (carbohydrate)
AA997902	Glucuronidase, beta	Metabolism (carbohydrate)
AI045939	transcribed sequence with weak similarity to protein sp:Q16706 (H.sapiens) M2A1_HUMAN Alpha-mannosidase II	(Metabolism)
AA963226	Defender against cell death 1	Anti-apoptosis
AA996844	ADP-ribosylation factor 4	Protein transporter activity
AA925933	cathepsin S	Proteolysis
AI060106	hepsin	Proteolysis, cell growth?
AI059256	zinc finger protein 36, C3H type-like 1	Transcription factor
AI058820	similar to Ras-related protein Rab-25 (LOC310632)	Ras-related
AA899453	similar to 60S ribosomal protein L23a (LOC289656)	Protein synthesis
AA956498	similar to Splicing factor, arginine/serine-rich 3 (LOC361814)	Unknown
Significant lower expression in wheat bran and benzylisothiocyanate group compared to control group		
AA924917	mitogen-activated protein kinase 12	Signal transduction
AA859805	similar to Loxl protein (LOC315714)	Unknown
AA963644	cDNA clone UI-R-E1-gg-e-05-0-UI 3'	Unknown

Similar to curcumin, differentially expressed genes in small intestinal scrapings in the wheat bran group were only found at week 7. Genes involved in metabolism, genes involved in ras-signaling and ribosomal genes were differentially expressed in response to wheat bran. In addition to the metabolism genes in table 4 expression of acetyl-Coenzyme A acyltransferase 2 (Acaa2), mannosyl (alpha-1,6-)-glycoprotein beta-1,2-N-acetylglucosaminyltransferase (Mgat2), mevalonate pyrophosphate decarboxylase (Mvd) and UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (Uae1) was upregulated by wheat bran. Genes involved in ras-signaling that were upregulated by wheat bran included a gene similar to ras-related protein Rab-25 (table 7.3), plysia ras-related homolog A2 (Arha2), Rho interacting protein 3 (Rhoip3), G protein-coupled receptor kinase 6 (Gprk6) and RAN, member of ras oncogene family (Ran). Several ribosomal genes were upregulated in small intestinal mucosa in response to wheat bran (figure 7.6). Similar to Arf4 (table 7.3) expression of ADP-ribosylation factor 1 (Arf1) was also upregulated in response to wheat bran. In addition to the immune-related genes in table 7.3, also expression of Ig delta heavy chain constant region and

of a gene similar to Fc fragment of IgG binding protein was upregulated in response to wheat bran. Expression of clusterin (Clu) and defender against cell death 1 (Dad1), both involved in apoptosis, was upregulated by wheat bran.

In the rutin diet group expression of 28 genes was upregulated in intestinal scrapings at week 26 (gene expression at week 7 could not be measured). Expression of several genes involved in proteolysis was induced, including proteasome subunit, alpha type 2 (Psma2), ubiquitin D (Ubd) and polyubiquitin (figure 7.7). Several sequences similar to genes with a role in gene transcription (e.g. sequence with strong similarity to PPAR binding protein) were upregulated. Also, expression of immune-related genes (beta-2-microglobulin and Fc receptor, IgG, alpha chain transporter) and signal transduction genes (e.g. p21 (CDKN1A)-activated kinase 2) was induced (data not shown).

The largest number of differentially expressed genes was found in the benzyl isothiocyanate group. Expression of two cytochrome c oxidase genes (subunit Va and subunit VIa) was upregulated at week 7 (figure 7.8). Expression of another cytochrome c oxidase gene (subunit Vb) was upregulated by wheat bran (figure 7.8). A considerable number of genes that were upregulated by benzyl isothiocyanate at week 7 encode proteins with oxidoreductase activity like the two cytochrome c oxidase genes. These genes are listed in table 7.4. Expression of prolyl 4-hydroxylase, like cytochrome c oxidase genes involved in electron transport, was also upregulated by benzyl isothiocyanate at week 7. Expression of genes involved in transport was upregulated at week 7, e.g. three solute carrier family genes (Slc10a1, Slc17a1, Slc25a5) and three ATPase or ATP synthase genes. In addition to the immune-related genes in table 7.3, benzyl isothiocyanate also upregulated expression of the immune-related genes secreted phosphoprotein 1 (Spp1), adipsin (And) and CD24 antigen (Cd24) at week 7.

A few genes were differentially expressed in the benzyl isothiocyanate group at both time points. Expression of a gene similar to ilvB (bacterial acetolactate synthase)-like isoform 1 was induced at week 7 and repressed at week 26 (figure 7.3). Expression of plasminogen activator urokinase (Plau) and of a gene similar to LOXL protein was downregulated by benzyl isothiocyanate at both time points. Expression of defender against cell death 1 (Dad1), aldehyde dehydrogenase family 3 subfamily A2 (Aldh3a2), serine threonine kinase pim3 (Pim3) and proteasome subunit beta type 1 (Psmb1) was upregulated at both time points.

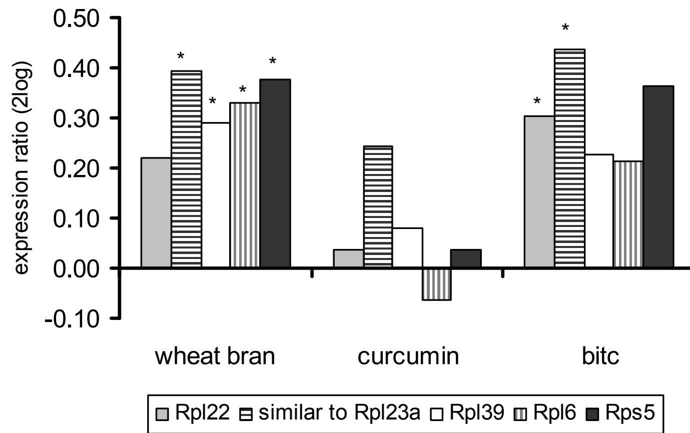

Figure 7.6. Expression changes of ribosomal genes in small intestinal scrapings from wheat bran, curcumin and benzyl isothiocyanate (bitc) diet groups at week 7. Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). Rpl22: ribosomal protein L22; Rpl23a: ribosomal protein L23a; Rpl39: ribosomal protein L39; Rpl6: ribosomal protein L6; Rps5: ribosomal protein S5.
* indicates significant difference in expression between diet group and control group.

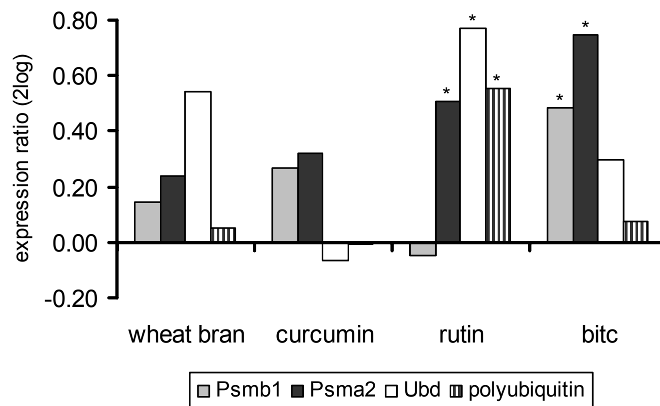

Figure 7.7. Expression changes of proteolysis genes in small intestinal scrapings from wheat bran, curcumin, rutin and benzyl isothiocyanate (bitc) diet groups at week 26. Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). Psmb1: proteasome subunit, beta type 1; Psma2: proteasome subunit, alpha type 2; Ubd: ubiquitin D.
* indicates significant difference in expression between diet group and control group.

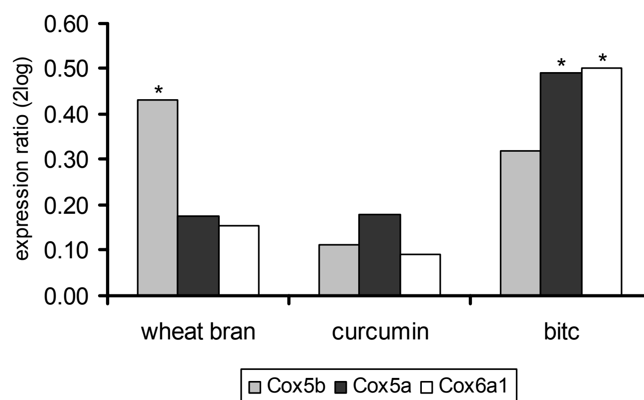

Figure 7.8. Expression changes of cytochrome c oxidase genes in small intestinal scrapings from wheat bran, curcumin and benzyl isothiocyanate (bitc) diet groups at week 7. Y-axis shows ^{2}log values of expression ratio (expression in diet group compared to expression in control group). Cox5b: cytochrome c oxidase, subunit Vb; Cox5a: cytochrome c oxidase, subunit Va; Cox6a1: cytochrome c oxidase, subunit VIa, polypeptide 1.
* indicates significant difference in expression between diet group and control group.

Table 7.4. Genes encoding proteins with oxidoreductase activity that were upregulated by benzyl isothiocyanate at week 7.

Accession Number	Gene Name	Gene Symbol
AA998201	cytochrome c oxidase, subunit Va	Cox5a
AA899832	cytochrome c oxidase, subunit VIa, polypeptide 1	Cox6a1
AA875268	similar to NADH dehydrogenase (ubiquinone) Fe-S protein 7 (LOC362837)	-
AA923966	aflatoxin B1 aldehyde reductase	Afar
AA900573	malate dehydrogenase 1	Mdh1
AA866390	retinol dehydrogenase type III	RoDH(III)
AI044102	Arachidonate 5-lipoxygenase	Alox5
AA956846	aldehyde dehydrogenase family 3, subamily A2	Aldh3a2
AA999026	prostaglandin-endoperoxide synthase 1	Ptgs1

At week 7, expression of two ribosomal genes was upregulated (figure 7.6). At week 26 however, expression of several ribosomal genes (ribosomal protein S2, ribosomal protein S14, similar to ribosomal protein S18 and similar to ribosomal protein L36a) was downregulated. At week 26 benzyl isothiocyanate induced expression of a number of protein kinase genes: protein kinase regulatory type 2 alpha (Prkar2a), protein kinase C delta (Prkcd), Pim3 and casein kinase 1 delta.

Discussion

Effects of the four plant compounds wheat bran, curcumin, rutin and benzyl isothiocyanate in intestinal tissues were studied using microarrays. AOM-treated rats were fed a diet with one of these compounds or a control diet for 8 months. At several time points intestinal tissues were collected to study colon tumor development. As an early putative preneoplastic biomarker for colon tumor development ACF were counted after 7, 15 and 26 weeks. After eight months tumor incidence, multiplicity and size was determined [20]. It was concluded that number of ACF at any of the intermediate time points was not predictive for colon tumor development [20]. In the same study, scrapings of the small intestinal mucosa were collected for measurement of gene expression profiles. Two approaches were chosen in the analysis of the gene expression data. Firstly, it was investigated by multivariate statistical analysis whether the gene expression profiles in small intestine measured at interim sections (at week 7 and 26) were predictive for colon tumor development after 8 months. In a few other microarray studies this multivariate statistical method, partial least squares, was also used in the data analysis, e.g. to identify genes with increased expression in specific cell cycle stages [24] or to predict clinical outcome of breast cancer [25]. Secondly, the gene expression changes in intestinal mucosa in response to each of the four compounds were analyzed individually and these gene expression changes were interpreted in relation to effects on gastrointestinal health, including intestinal cancer, inflammatory diseases and oxidative stress. At the end of the study (after 8 months) colon tumor multiplicity was significantly lower in the curcumin and the wheat bran group, compared to the control group, demonstrating a protective effect of curcumin and wheat bran on development of colon cancer. Therefore, colon tumor multiplicity was chosen as a parameter for colon carcinogenesis in the correlation analysis. For both time points (week 7 and week 26) a subset of genes with a significant difference in expression across the five diet groups was selected from the expression dataset of 3000 rat genes. Multivariate statistical analysis revealed a good correlation between the gene expression profiles of the subset of genes at both time points and tumor multiplicity, indicating that gene expression profiles measured in small intestine might be predictive for processes related to colon carcinogenesis and that effects in small intestine and colon are related. This observation is supported by other correlations between effects in small intestine and colon described previously, e.g. the observation that increased detoxification of carcinogens by upregulation of drug metabolizing enzyme activity in small intestine is related to prevention of colon cancer development [26]. Cancer-preventive or -reducing effects of plant compounds in the colon can be exerted from both the luminal and the basolateral side, i.e. before and after absorption. Thus, absorption and metabolism in small intestine or metabolism in the liver could be related to preventive effects of a specific compound on colon cancer development. Furthermore, nutrient-gene interactions in the small intestine (as described in this study) may result in some sort of signaling towards colonic epithelium.

Effects on immune- or inflammation-related genes in small intestine could also be related to colon carcinogenesis since chronic inflammation of intestinal tissue is a risk factor for development of colon cancer [27]. The evidence for a link between effects in small intestine and effects in colon as described above is strengthened by the correlation of small intestinal gene expression profiles with colon tumor development.
In the present study, all compounds exert an effect on immune-, anti-inflammatory- and anti-oxidative stress-related genes, which could be interesting with respect to inflammatory diseases of the intestine. It was previously reported that dietary fiber and short chain fatty acids could exert effects on immune-related processes in the intestine [28, 29]. Curcumin is known to be a potent anti-inflammatory agent [12, 30]. In addition to its cancer-preventive effects, curcumin can also inhibit development of inflammatory bowel disease [31]. Anti-inflammatory effects have also been reported for rutin [32] and benzyl isothiocyanate [33]. Possibly, these immune-related effects of food compounds in small intestine could present an additional way of exerting effects in colon, mediated by (anti-)inflammatory signals as some kind of messenger.

Although biomarker discovery through gene expression profiling is promising, the relatively large amount of tissue that is needed for gene expression profiling may limit the use of this technique. However, in combination with RNA amplification techniques, it will be possible to measure gene expression profiles in much smaller amounts of tissue or even single intestinal crypts.

A number of the functional groups of differentially expressed genes that contributed most to the predictive value of gene expression profiles at week 7 are known to play a role in colon carcinogenesis, for example genes involved in cell growth and cell death. In intestinal tissues a constant cell number is maintained by the balance between cell growth and cell death [34, 35]. A disturbance of this balance can lead to hyperproliferative epithelium, one of the first steps in carcinogenesis [36]. Cytoskeleton genes could be involved since they play a role in cell cycle control. Ribosomal genes could also play a role in colon carcinogenesis since many studies reported that expression of ribosomal genes is higher in human colon cancer tissue compared to normal colon tissue [37-39]. Expression profiles of a number of ribosomal genes correlated positively with colon tumor multiplicity and in addition a number of ribosomal proteins were differentially expressed in the wheat bran and the benzyl isothiocyanate group, compared to the control group. A large number of genes related to oxidative stress or involved in oxidoreductase activity contributed to the correlation of gene expression profiles at week 7 with colon tumor multiplicity. Expression of one of these genes, metallothionein, was found to be downregulated in human colon cancer tissue compared to normal colon tissue in a

number of studies [40, 41](Chapter 4). As mentioned previously, oxidative stress can also play a role in intestinal inflammation.

In the rutin group expression of a number of genes involved in proteolysis or protein degradation was higher compared to the control group. Protein degradation by proteasomes plays a role in cell cycle control and apoptosis [42], indicating that these genes could also be involved in colon carcinogenesis. The ubiquitin-proteasome system, that controls protein degradation, is also indicated to play a role in the process of gene transcription [43, 44]. Correspondingly, expression of ubiquitin-protease genes and a number or transcription factors was upregulated in the rutin group at week 26.

In addition to functional groups of genes described above, some other colon cancer-related genes were differentially expressed in intestinal mucosa. Expression of plasminogen activator urokinase (Plau) was lower in the benzyl isothiocyanate group compared to the control group at both time points. Plau could play a role in tumor cell invasion and was reported to be involved in colon carcinogenesis [45]. It has previously been reported that butyrate downregulated expression of Plau in colon cancer cells [46]. Wendum et al. reported that Pla2g2a, an acute phase protein that is involved in inflammatory diseases [47], was expressed at a high level in tumors of the colon and the small intestine [48]. In our study expression of Pla2g2a was upregulated by wheat bran and downregulated by curcumin at week 7. Expression of beta glucuronidase was upregulated by wheat bran and benzyl isothiocyanate. This might be related to colon cancer development since bacterial beta glucuronidase activity in colon could play a role in AOM-induced colon carcinogenesis [49].

In general, the largest number of differentially expressed genes in the diet groups compared to the control group was found at week 7. Since biomarkers are most relevant when they can be measured at early time points, we focused on expression changes at week 7. In addition, at week 26 no significantly differentially expressed genes were found in response to the wheat bran and curcumin diets, the two diets that exerted a protective effect on colon tumor development as measured after 8 months. Similarly, Madar et al. found no effect of different dietary fibers on morphology in ileum of dimethylhydrazine-treated rats after 24 weeks [50]. The limited effect of curcumin on gene expression in intestinal mucosa could perhaps be related to the low rate of metabolism of curcumin in rat intestine compared to human intestine [51].

In conclusion, it was found that expression profiles of a subset of differentially expressed genes in small intestinal tissue at two intermediate time points (week 7 and 26) were predictive for colon tumor multiplicity after 8 months. This observation supports other findings indicating that effects in small intestine could be connected to or important for effects in colon. Some of the (functional groups of) genes that contribute to the predictive value of gene expression profiles at week 7 or that were differentially expressed in rats fed a diet with a certain plant compound were indicated in other studies to play a role in colon

carcinogenesis. Effects on immune-related genes could indicate an effect on colon cancer through modulation of inflammatory processes and could indicate an effect on intestinal health in general. Thus gene expression profiling of intestinal tissue could be useful in the search for biomarkers for effects of food compounds related to intestinal cancer development and intestinal health e.g. by (anti-) inflammatory effects.

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Summary & Future perspectives

Summary of results

Traditionally, gene expression measurements were restricted to single or small numbers of genes using e.g. reverse transcription PCR or Northern blots. Recently, methods for large-scale gene expression measurements have radically changed this. Microarrays, which allow for expression measurements of an ever-increasing number of genes, approaching 'whole genome' expression analysis, are currently being used in many areas of research. In this thesis large-scale gene expression measurement was applied in the field of colon cancer prevention by food compounds. Effects of a range of phytochemicals (quercetin, rutin, curcumin, resveratrol, wheat bran and benzyl isothiocyanate) on gene expression in intestinal cells were measured with microarrays.

In **chapters 2-5** human cultured colon (cancer) cells are used for large-scale gene expression studies. First, effects of quercetin, an abundant flavonoid present in e.g. apples and onions, on gene expression in Caco-2 cells were measured after exposure of the cells for 48 hours (**chapter 2**). A different set-up was chosen in the second study, gene expression changes were measured in HT29 cells in response to curcumin exposure for 3, 6, 12, 24 and 48 hours. Gene expression changes after 3 and 6 hours of exposure to curcumin were also measured in Caco-2 cells. By studying gene expression changes at many time points, both genes that responded at early time points and genes that responded at later time points were identified (**chapter 3**). It was found that the response to curcumin in HT-29 and Caco-2 cells was partly overlapping, but differences in the gene expression profiles were also observed. In **chapter 4** expression profiles of 14 different human cell lines derived from human colon tissue were compared, representing various stages of malignancy observed during colon carcinogenesis. In addition, by comparing expression profiles of colon biopsies from tumor and normal tissue a subset of colon cancer-specific genes was identified. This subset was used to study differences in expression in the panel of colon cell lines, thereby serving as a biomarker set derived from *in vivo* samples. Using this approach, *in vitro* models were connected to the *in vivo* situation. Clear differences were observed between the expression profiles of the various cell lines. Next, two cell lines from the panel were chosen for screening of effects of food compounds. Effects of quercetin, curcumin and resveratrol were measured in HT29 and T84 cells after 2, 6 or 24 hours (**chapter 5**). Some (functional groups of) genes were affected by all three compounds. Also, differences in response between the cell lines and in effects of the compounds were found.

The mechanisms of cancer prevention by food compounds (including quercetin, curcumin and resveratrol) described mostly in literature include inhibition of cell proliferation and induction of apoptosis. Correspondingly, all of the compounds tested in our studies had an effect on cell cycle genes in one or more of the cell lines. The effect of quercetin on expression of cell cycle genes in Caco-2 cells corresponded to a small but significant inhibition of proliferation of

Caco-2 cells at low concentrations, at which no cytotoxicity could be observed (**chapter 2**). The effect of curcumin on cell cycle genes in HT29 cells coincided with a cell cycle arrest in the G2/M phase already after 3 hours of exposure (**chapter 3**). Effects on apoptosis were much less pronounced in our studies. Probably, effects on apoptosis are more likely to occur at protein level than RNA level [1]. Also, expression of several colon cancer-related genes was affected by the compounds, e.g. downregulation of expression of p53 in HT29 cells. Other groups of genes that were differentially expressed in response to one or more of the compounds included proteasome genes, DNA repair genes, genes involved in signal transduction pathways, genes involved in cell adhesion, genes involved in transcription. All these processes could play a role in the colon cancer-preventive action of the compounds studied.

In **chapter 2 and 3**, microarray results were validated by real-time RT-PCR for a number of genes. Although normalization methods were different for microarray data and RT-PCR data, in most cases microarray results were confirmed by real-time RT-PCR. Whereas microarray data are normalized in a global way (using data for all genes measured), RT-PCR data were normalized for expression of one housekeeping gene (being beta-actin or GAPD).

Chapter 6 and 7 describe the results of an *in vivo* study using a rat model. A traditional approach of studying colon cancer prevention (using the azoxymethane (AOM) model) was combined with (large-scale) gene expression measurement. The effects of wheat bran, curcumin, rutin and benzyl isothiocyanate on colon carcinogenesis were studied in AOM-treated rats. Colon tumor multiplicity was significantly lower in the wheat bran and curcumin group compared to the control group. Number or size of aberrant crypt foci (ACF) after 7, 15 and 26 weeks were not predictive of colon tumor development after 8 months (**chapter 6**). Gene expression of a number of colon cancer genes was measured in colon tumors of the different diet groups. When gene expression in tumors was correlated to colon tumor multiplicity, the highest correlation was found for TIMP-1 (possibly involved in tumor invasion) and p53 (tumor suppressor gene and transcription factor involved in cell cycle control) (**chapter 6**). At two intermediate time points (week 7 and week 26) scrapings of small intestinal mucosa were collected. Gene expression profiles were measured in these scrapings (**chapter 7**). Both at week 7 and at week 26, high correlation was found between expression profiles of a subset of about 400 differentially expressed genes and colon tumor multiplicity after 8 months. Moreover, functional groups of genes involved in colon cancer development were differentially expressed in specific diet groups compared to the control group and/or contributed to the predictive value of the gene expression profiles, e.g. genes involved in cell growth and cell death, ribosomal genes, immune-related genes. This indicates that these small intestinal gene expression profiles could be predictive of colon tumor development and it demonstrates that effects in small intestine and colon are related. Effects

of individual compounds were also studied and there were differences in effects of the compounds on gene expression in the small intestinal tissue. Wheat bran and curcumin only affected gene expression at week 7. In general, the largest response was found at week 7 and the benzyl isothiocyanate diet resulted in the largest number of differentially expressed genes in small intestinal scrapings compared to the control group. It was concluded that gene expression profiling of small intestinal tissue could be useful in the search for reliable biomarkers of effects of food compounds related to protection against intestinal cancer development and promotion of intestinal health.

In the last five years, the microarray technology developed quickly, worldwide and also in our laboratory. As an example, the research described in this thesis was started with a set of 4000 human cDNAs that were first multiplied by PCR, checked by gel electrophoresis (for presence of a single product) and purified before spotting on microarrays. Towards the end of the project the microarrays used contained larger sets of oligos (17000, ready-to-spot), together with a large number of control spots. Similarly, labeling and hybridization protocols were improved over time.

Transcriptomics in nutritional research - potential and pitfalls

Altogether, the studies in this thesis show the potential of large-scale gene expression profiling techniques in nutritional research. In our studies and other microarray studies in nutrition interesting results useful for possible new biomarker discovery ranged from single novel genes to groups of genes or specific pathways. Several of these pathways or processes were already reported in other studies to play a role (e.g. cell cycle arrest or inhibition of cell growth), which validates these microarray studies. Furthermore, new and interesting leads, like e.g. effects on proteasome genes, tubulin genes and immune-related genes, were found by studying expression of thousands of genes. The microarray studies are thus hypothesis-generating as well as hypothesis-confirming.

Although the studies yielded interesting results, there are still many limitations in microarray studies. Analysis and interpretation of the huge amounts of expression data resulting from microarray studies is currently still difficult and time-consuming. In many studies, the process of microarray data analysis ends when a list of differentially expressed genes has been generated. A selection of these genes is published and related to what we already know. However, publishing lists of differentially expressed genes should not be the end point of data analysis. The full power of the technique can be exploited only when data analysis is extended with more sophisticated analysis methods, looking e.g. at interactions between genes in pathways or at transcription factors controlling expression of genes. It is expected that the field of bioinformatics, focusing on microarray data analysis and interpretation, will mature and help to facilitate microarray data analysis and interpretation, which will then make it

feasible to analyze data at this level. Pathway analysis, including visualization of gene expression changes in pathways and combining different pathways, is a very important part of this process. Mainly during data analysis of the study in **Chapter 3** GenMAPP [2] was used to visualize gene expression changes. This resulted e.g. in a clear overview of expression changes in different phases of the cell cycle in cells treated for different time periods, which could be used for comparison to effects on cell cycle distribution as measured by flow cytometry.

Statistical analysis of microarray data is largely dependent on design of the experiment. This indicates the importance of careful experimental design, including number of replicate arrays and the choice between e.g. dye-swap design or common reference design without dye-swap. Many reports address this issue [3, 4]. In **chapter 2, chapter 4** (biopsy data) and **chapter 7**, more than 2 expression ratios were calculated for each gene, therefore statistical analysis was more easily applicable. In these studies the SAM (Statistical Analysis of Microarrays) tool [5] was used together with other methods. Multiple testing, as for thousands of genes in microarray data, can yield large numbers of false positives. SAM, an easy-to-use Excel plug-in, gives an estimate of false discovery rate by permutation of the data and allows the user to set a threshold for false discovery rate. It was most useful when comparing expression profiles between two groups (e.g. treated and control). When comparing more than two groups, principal component analysis was found to be very useful. By using this multivariate statistical technique a two- or three-dimensional visualization of the samples can be created in which distance between samples is a measure of similarity or dissimilarity of gene expression profiles in the samples (**chapter 3-5**). In addition, subsets of genes that contribute to differences between samples can be extracted from principal component analysis, like e.g. in **Chapter 4**, where gene expression profiles of 14 human colon cell lines were compared. In **Chapter 7** a different multivariate statistical tool was used (partial least squares) to correlate gene expression profiles to a clinical outcome, in this case colon tumor multiplicity. This yielded interesting results in our study and this could be a valuable data analysis approach in biomarker discovery using gene expression profiling.

Although the humane genome has been sequenced, the annotation of the genes is a continuing process. This means that probe allocation and gene names can still include a considerable amount of mistakes and researchers need to update information on the genes present on the arrays regularly. A very important aspect is a uniform naming of genes and their function. This is currently being addressed by the Gene Ontology consortium (www.geneontology.org). A number of tools that add gene ontology information to a list of genes are available at the Internet. In addition, some of these programs also contain a possibility for significance analysis of functional groups of genes (i.e. to assess whether significantly more genes in a specific functional group are differentially expressed than would be expected by chance) [6, 7]. This kind of tool is very useful to get a quick overview of the pathways or processes

affected and was used in **Chapter 5**. In addition, information on genes was collected from Internet databases like NCBI's LocusLink and Unigene (http://www.ncbi.nlm.nih.gov/), GeneCards (http://bioinformatics.weizmann.ac.il/cards/) and Stanford's SOURCE (http://source.stanford.edu/cgi-bin/source/sourceSearch).

Furthermore, also in nutrition research standardization of microarray experiments and data handling is an important issue [8]. When microarray data are generated in a more standardized way and are deposited in databases, data can be more easily shared and compared. For studies described in this thesis preprocessing of the microarray data was mostly similar: excluding genes that were automatically or manually flagged by Imagene (the data extraction program), setting a threshold for signal-to-background ratio and intensity-dependent Lowess data normalization [9]. The process of data analysis and interpretation that followed the data preprocessing differed between the studies as indicated in the respective chapters and in this paragraph.

In conclusion, data analysis of large-scale gene expression profiling studies is a potentially powerful approach, if study design and data analysis strategy are sound, but is a largely underestimated task. At the moment many helpful tools are being developed and are becoming available on the Internet. However, in addition to adequate analysis tools, researchers also need to adjust their way of thinking. Since thousands of genes are being studied, researchers in this field, instead of gaining in-depth knowledge on one pathway, will need to gain knowledge on many processes and pathways. Therefore, researchers in nutrigenomics will need to collaborate more and profit from each other's knowledge. In a European context this kind of collaboration was started in the European NutriGenomics Organization (www.nugo.org), a network of more than 20 research groups working in the field of nutrigenomics.

Future nutrigenomics perspectives

The term nutrigenomics, or nutritional genomics, applies to research into genome-wide effects of nutrition, e.g. the influence of nutrients on expression levels of all genes, proteins and metabolites [10-12]. Research into effects of individual genetic differences on response to nutrition is referred to as nutrigenetics. In the studies described in this thesis large-scale gene expression measurement (referred to as transcriptomics) was used to provide insights into molecular effects of various plant compounds in relation to colon cancer. Models used were cultured colon cancer cells and an animal model. These cultured colon cancer cells are widely used in research. However, these cancer cells represent advanced stages of carcinogenesis since they originate from colon tumors or metastases of colon tumors (**chapter 4**). As nutrition or food compounds are likely to play a role mainly in prevention of carcinogenesis and not regression of already present tumors, it would seem logical to use cells from an earlier stage of colon carcinogenesis in these studies. In actual practice, it is very difficult to grow

cells from normal tissue (like NCM460 in **chapter 4**) outside the body. For this reason, effects of quercetin, curcumin and resveratrol (although planned) could not be measured in NCM460 cells. Also, primary human cells or human cells from early disease stages are not easily available. Blood samples, on the other hand, are often collected during human dietary intervention studies. Gene expression profiles can be measured in these samples (lymphocytes), as well as protein and metabolite profiles [10, 13]. Possibly, transcriptome and proteome patterns in white blood cells or proteome and metabolome patterns in plasma can serve as biomarkers or predictors for effects of food compounds in relation to health and early phases of disease. Overall, choice of model systems in cancer prevention research deserves more attention, since it would be extremely valuable to include models of early stages of (colon) carcinogenesis in nutrition research.

Concerning rat or human studies, a promising method is collection of specific cell types from human or animal tissues by laser microdissection which can now be used in combination with large-scale gene expression measurement [14]. Figure 8.1 shows an example of laser microdissection of colonic crypts from a rat colon tissue section. Small amounts of RNA isolated from samples collected by microdissection can be amplified to yield a sufficient amount of RNA for microarray analysis. Several RNA amplification techniques have been described, compared and are being validated [15, 16].

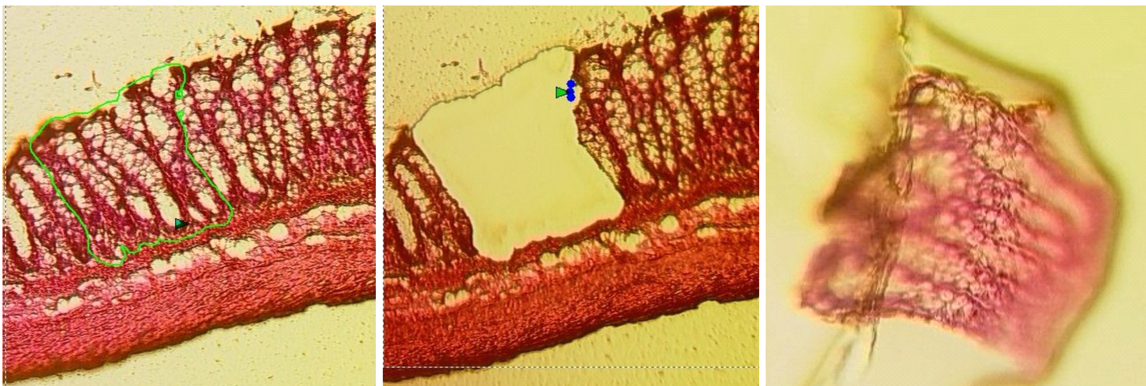

Figure 8.1. Laser microdissection of colonic crypts from a frozen tissue section of rat colon (8 μm).

Large-scale gene expression measurement is only one of the approaches in the field of nutrigenomics. Effects of nutrition can also be measured at the level of proteins or metabolites, with large-scale techniques like 2D gel electrophoresis (proteins) and mass spectrometry or nuclear magnetic resonance (NMR) (metabolites). Combination of large-scale gene expression profiling with proteomics and/or metabolomics together with studies on intestinal metabolism and absorption will help to get a more complete picture of the effects, mechanisms and potential of food compounds in all kinds of health-related processes. This holistic approach is also called 'systems biology' [17].

Obviously, food compounds do not exert their effects through one mechanisms of action but through multiple mechanisms in parallel or a cascade of actions. Also, people do not consume single food compounds, but foods that consist of many different (bioactive) compounds. Therefore, studying changes in expression of thousands of genes, proteins and metabolites will be useful to elucidate mechanisms of action of foods or specific food compounds, as is shown by the studies in this thesis. The wealth of data from nutrigenomics studies can yield valuable information on many diet-related diseases like cancer, cardiovascular diseases, diabetes, obesity etcetera. The challenge of the next years is to facilitate analysis and get more experienced in interpretation of these large amounts of data. Only then, the large potential of nutrigenomics can become reality. Hopefully, this will then ultimately lead to discovery of biomarkers and mechanisms of early stages of disease onset, relevant in prevention of disease by nutrition.

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NEDERLANDSE SAMENVATTING

Inleiding

Het ontstaan van kanker is sterk gerelateerd aan omgevingsfactoren. Voeding en voedingsstoffen zijn daarvan een belangrijk voorbeeld, met name bij kanker in het maagdarmkanaal. Dikkedarmkanker is een van de meest voorkomende soorten van kanker in westerse landen. Algemeen wordt aangenomen dat voeding een grote rol kan spelen bij de bescherming tegen het ontstaan van dikkedarmkanker. In verschillende epidemiologische studies is een beschermend effect van vooral groente en fruit aangetoond. Een aantal groepen van stoffen uit groente en fruit, die een rol kunnen spelen bij dit beschermende effect, zijn geïdentificeerd, maar er is weinig bekend over de mechanismen van de beschermende werking van deze stoffen tegen dikkedarmkanker.

In de studies beschreven in dit proefschrift werd onderzoek gedaan naar de mechanismen achter de mogelijk beschermende werking van een aantal voedingsstoffen uit planten tegen het ontstaan van dikkedarmkanker. Dit werd gedaan door in darmcellen de activiteit van de genen (genexpressie) te meten, dus door het meten van de hoeveelheid messenger RNA (mRNA) die is afgelezen van de onderzochte genen in de darmcellen. De techniek die hiervoor gebruikt werd is de zogenaamde 'microarray' techniek. Met deze techniek is het mogelijk om de genexpressie van duizenden genen tegelijkertijd te meten, in tegenstelling tot andere technieken waarmee slechts de expressie van één of enkele genen gemeten kan worden, zoals RT-PCR ('reverse transcription – polymerase chain reaction'). Door expressie-veranderingen van duizenden genen te meten in darmcellen kan het effect van een voedingsstof in zijn volle omvang bestudeerd worden en op deze manier zou het werkingsmechanisme met betrekking tot bescherming tegen kanker ontrafeld kunnen worden. Dit soort onderzoek wordt ook wel 'nutrigenomics' genoemd.

De voedingsstoffen uit planten die gebruikt werden in de studies zijn quercetine (o.a. aanwezig in appels en uien), curcumine (koenjit of curcuma, kruid uit de Aziatische keuken), resveratrol (o.a. aanwezig in druiven en rode wijn), tarwevezels (uit granen) en benzyl isothiocyanaat (o.a. aanwezig in broccoli en spruitjes). Uit studies met darmkankercellen en ratten is gebleken dat deze stoffen inderdaad een beschermend effect zouden kunnen hebben tegen het ontstaan van darmkanker.

Dit proefschrift beschrijft twee soorten studies. De eerste hoofdstukken beschrijven de zogenaamde 'in vitro' studies, waarbij gebruik werd gemaakt van humane darmkankercellen (die buiten het lichaam gekweekt kunnen worden) om het effect en de werkingsmechanismen van de voedingsstoffen te bestuderen. In de laatste hoofdstukken worden de resultaten van de dierstudie ('in vivo' studie) besproken, waarbij gebruik werd gemaakt van een rat model voor dikkedarmkanker.

In vitro studies
In de eerste studie (hoofdstuk 2) werden humane dikkedarmkankercellen (Caco-2 cellen) gedurende 48 uur blootgesteld aan quercetine, waarna veranderingen in de expressie van 4000 humane genen gemeten werden. In de tweede studie (hoofdstuk 3) werd voor een andere opzet gekozen en werden genexpressie-veranderingen in de tijd gevolgd. Humane dikkedarmkankercellen (HT29 cellen) werden blootgesteld aan curcumine en expressie-veranderingen van 4000 humane genen werden gemeten na 3, 6, 12, 24 en 48 uur blootstelling. Op deze manier konden zowel genen die snel reageerden (binnen 3 of 6 uur) als genen die op latere tijdstippen reageerden geïdentificeerd worden. Genexpressie-veranderingen na 3 en 6 uur blootstelling werden ook gemeten in Caco-2 cellen behandeld met curcumine. In Caco-2 en HT29 cellen werden zowel overeenkomsten als verschillen in respons op de blootstelling aan curcumine gevonden.
Naast Caco-2 en HT29 zijn er nog een groot aantal andere dikkedarmkanker-cellijnen. In hoofdstuk 4 werd de expressie van 4000 humane genen in 14 verschillende darm(kanker)-cellijnen gemeten en vergeleken. Er werden duidelijke verschillen tussen de genexpressie-profielen van de verschillende cellijnen waargenomen. Behalve in dikkedarm(kanker)-cellijnen werd de expressie van 4000 genen ook gemeten in humane dikkedarmtumoren en normaal humaan dikkedarmweefsel (biopten van dikkedarmweefsel werden verkregen via het Universitair Medisch Centrum St. Radboud te Nijmegen). De genen die significant hoger of lager tot expressie kwamen in humane dikkedarmtumoren vergeleken met normaal humaan dikkedarmweefsel werden geselecteerd als subset van dikkedarmkanker-specifieke genen. Deze subset van genen (gedefinieerd met behulp van de humane dikkedarmbiopten) werd vervolgens gebruikt om de verschillen tussen de 14 humane darm(kanker)-cellijnen te bestuderen. Uiteindelijk werden 2 cellijnen gekozen om effecten van voedingsstoffen te gaan bestuderen. Deze cellijnen (HT29 en T84) werden gedurende 2, 6 en 24 uur blootgesteld aan quercetine, curcumine of resveratrol, waarna de expressie van 17.000 genen gemeten werd (hoofdstuk 5). Expressie van een aantal (functionele groepen van) genen werden door zowel quercetine, curcumine als resveratrol beïnvloed. Daarnaast waren er ook duidelijke verschillen in respons tussen de twee cellijnen en binnen een cellijn tussen de voedingsstoffen.

De meest beschreven mechanismen van bescherming tegen het ontstaan van kanker door voedingsstoffen (zoals quercetine, curcumine en resveratrol) zijn remming van celdeling en inductie van apoptose (geprogrammeerde celdood). Normaal gesproken is er in darmepitheel een nauwkeurige balans tussen celdeling en celdood. Bij het ontstaan van darmkanker is deze balans verstoord en kunnen tumorcellen overleven en blijven delen. In overeenstemming met de reeds bekende mechanismen van bescherming hadden alle stoffen in onze studies een effect op genen betrokken bij celdeling en de celcyclus in één of meer cellijnen. Quercetine

had niet alleen een duidelijk effect op genen betrokken bij de celdeling, maar het zorgde ook voor een remming van de celdeling (hoofdstuk 2). Het effect van curcumine op genen betrokken bij de celdeling ging samen met een cel cyclus arrest in de G2/M fase (hoofdstuk 3). In tegenstelling tot effecten op genen betrokken bij celdeling werden effecten op genen betrokken bij apoptose veel minder gevonden. Waarschijnlijk wordt het proces van apoptose meer op het eiwit-niveau dan op het RNA-niveau beïnvloed.

De geteste stoffen hadden ook een effect op de expressie van genen waarvan bekend is dat ze een rol spelen bij het ontstaan van dikkedarmkanker, zoals p53. Andere groepen genen waarvan de expressie veranderde door blootstelling aan een of meer voedingsstoffen waren proteasoom genen, DNA herstel genen, genen betrokken bij signaal transductie, genen betrokken bij cel adhesie en genen betrokken bij transcriptie. Deze processen zouden allemaal een rol kunnen spelen bij de beschermende werking van quercetine, curcumine en resveratrol tegen het ontstaan van kanker.

In vivo studie

Aan het begin van de dierstudie werden alle ratten behandeld met azoxymethaan (een kankerverwekkende stof), waardoor ze dikkedarmtumoren gingen ontwikkelen. Dit is een veel gebruikt model om het effect van (voedings)stoffen op het ontstaan van dikkedarmkanker te bestuderen. De ratten kregen gedurende 8 maanden een controle voeding of een voeding met tarwevezel, curcumine, rutine of benzyl isothiocyanaat. Alleen bij de ratten die de voeding met tarwevezel of curcumine kregen werd een beschermend effect gevonden. Het aantal dikkedarm-tumoren na 8 maanden was namelijk significant lager dan in de ratten die de controle voeding kregen. Er werd niet alleen na 8 maanden naar ontwikkeling van tumoren gekeken, maar ook werden op eerdere tijdstippen (na 7, 15 en 26 weken) 'aberrante crypt foci (ACF)', een mogelijk voorstadium van dikkedarmtumoren, geïnventariseerd. De effecten van de verschillende voedingsstoffen op aantal of grootte van ACF bleken echter niet voorspellend te zijn voor het effect op ontwikkeling van dikkedarmkanker na 8 maanden (hoofdstuk 6). In de verzamelde tumoren uit de verschillende groepen (controle, tarwevezel, curcumine, rutine of benzyl isothiocyanaat) werd de expressie gemeten van een aantal genen die betrokken zijn bij het ontstaan van dikkedarmkanker. Expressie van TIMP-1 (mogelijke rol in tumor invasie) en p53 (tumorsuppressor-gen en transcriptiefactor betrokken bij celdeling) in tumoren correleerde het beste met het aantal dikkedarmtumoren.

Op twee tijdstippen, 7 en 26 weken na de start van de dierstudie, is naast het dikkedarm-weefsel ook dunnedarmweefsel verzameld. Expressie van 3000 rat genen werd gemeten in dit dunnedarmweefsel. Voor ongeveer 400 genen werden significante verschillen in expressie gevonden tussen de 5 groepen (controle, tarwevezel, curcumine, rutine, benzyl isothiocyanaat). Op beide tijdstippen was er een hoge correlatie tussen het expressie-profiel van de subgroep van genen (met significante verschillen in expressie) en het aantal

dikkedarmtumoren. Dit geeft aan dat het expressie-profiel van een set genen die differentieel tot expressie komen in de dunne darm voorspellend zou kunnen zijn voor het ontstaan van dikkedarmtumoren. De voedingsstoffen hadden verschillende effecten op genexpressie in de dunne darm. Het grootste effect werd gevonden in week 7. De voeding met benzyl isothiocyanaat resulteerde in het grootste aantal significante genexpressie-veranderingen in de dunne darm. Verschillende (groepen van) genen betrokken bij het ontwikkelen van dikkedarmkanker droegen bij tot de voorspellende waarde van de expressie-profielen of hadden in een bepaalde groep een significant veranderde expressie ten opzichte van de controle groep. Genexpressie-profielen van darmweefsel kunnen dus nuttig zijn bij het zoeken naar biomarkers voor effecten van voedingsstoffen in relatie tot het beschermen tegen de ontwikkeling van darmkanker.

Gebruik van microarrays in voedingsonderzoek: mogelijkheden en uitdagingen

De studies beschreven in dit proefschrift illustreren de potentie van het gebruik van genexpressie-metingen op grote schaal in voedingsonderzoek. Een deel van de beschreven resultaten was in overeenstemming met resultaten beschreven in de literatuur, wat beschouwd kan worden als validatie van de microarray techniek. Daarnaast werden ook resultaten gevonden die nieuw inzicht geven in de effecten en werkingsmechanismen van de voedingsstoffen.

Ondanks dat het gebruik van microarrays redelijk is ingeburgerd, zijn er nog veel beperkingen aan het gebruik van microarrays in (voedings-)onderzoek. Analyse en interpretatie van de data is een moeilijke en tijdrovende taak, met name door de grote hoeveelheid data die zelfs binnen één experiment gegenereerd wordt. Het is de verwachting dat de zogenaamde bio-informatica een belangrijke rol gaat spelen bij de data-analyse en -interpretatie. Toegepaste analyse-programma's zijn nodig om de volledige potentie van microarrays te benutten. Een aantal specifieke 'tools', die al beschikbaar zijn via Internet, zijn gebruikt bij de analyse van de data in dit proefschrift, zoals een 'pathway' analyse programma (GenMAPP) voor het visualiseren van genexpressie-veranderingen in biochemische en metabole processen en een Excel 'plug-in' tool voor statistische analyse (SAM). Ook is regelmatig gebruik gemaakt van multivariate statistische analysemethoden ('Principal Components Analysis'). Een ander belangrijk punt bij microarray data-analyse is de naamgeving en de functie van de genen op de microarray. Hoewel de sequentie van het humane genoom bekend is, is van veel genen geen functie bekend. Verschillende databases op Internet zijn gebruikt om informatie over genen te verkrijgen. Naast ontwikkeling van data-analyse programma's en beschikbaarheid van Internet databases is het ook belangrijk dat onderzoekers hun manier van denken aanpassen aan de nieuwe technieken. Het bestuderen van duizenden genen tegelijkertijd betekent dat een onderzoeker kennis over veel verschillende processen en 'pathways' moet hebben of krijgen. Het is daarom belangrijk dat onderzoekers meer gaan samenwerken en gaan profiteren van

elkaar's kennis. In Europa is recent de Europese Nutrigenomics Organisatie (www.nugo.org) opgezet, een netwerk van meer dan 20 onderzoeksgroepen die samenwerken in het veld van nutrigenomics.

Het is het meest waarschijnlijk dat voeding een rol speelt in de preventie van het ontstaan van kanker, dus in een vroeg stadium van het ontwikkelen van een tumor. In dit opzicht zou het dus logisch zijn om een model te gebruiken van een zo vroeg mogelijk stadium van dikkedarmkanker. In praktijk is het erg moeilijk om dit soort humane cellen voor onderzoek te gebruiken en daarom worden humane cellen afkomstig van een verder gevorderde tumor gebruikt. Het is wel mogelijk om grootschalige expressie-metingen te doen in bloedmonsters. Deze monsters zijn eenvoudiger te verkrijgen en worden bovendien vaak verzameld tijdens humane voedingsinterventiestudies. Mogelijk kunnen expressie-profielen gemeten in bloedmonsters in verband gebracht worden met gezondheid en vroege stadia van ziekten.
Een veelbelovende techniek die gebruikt kan worden in combinatie met microarrays is laser-microdissectie. Met behulp van laser-microdissectie kunnen specifieke celtypen geïsoleerd worden uit een monster; bijvoorbeeld uit een stukje dikkedarm van een rat kunnen specifiek de dikkedarmcrypten verzameld worden. Na vermenigvuldiging (amplificatie) van het mRNA als extra tussenstap (vanwege de geringe hoeveelheid materiaal die bij deze techniek beschikbaar is) kan dan expressie van duizenden genen gemeten worden met de microarray techniek.
Effecten van voedingsstoffen kunnen niet alleen op genexpressieniveau gemeten worden, maar ook op het niveau van eiwitten of metabolieten. Methoden voor grootschalige meting van eiwitten ('proteomics') en metabolieten ('metabolomics') zijn respectievelijk de 2D-gelelectroforese en massa spectrometrie of NMR ('Nuclear Magnetic Resonance'). Door het combineren van meten van expressie van duizenden genen met proteomics en/of metabolomics en met studies naar absorptie en metabolisme kan een completer beeld worden verkregen van effecten, mechanismen en potentie van voedingsstoffen in allerlei aan gezondheid gerelateerde processen. Deze holistische aanpak wordt ook 'systeembiologie' genoemd ('systems biology').

Het is duidelijk dat voedingsstoffen effect uitoefenen via verschillende mechanismen of via een cascade van acties. Bovendien consumeren mensen geen losse voedingsstoffen, maar voedsel dat bestaat uit een complex mengsel van vele bioactieve stoffen in een ingewikkelde matrix. Het bestuderen van veranderingen in expressie van duizenden genen, eiwitten of metabolieten is dus erg waardevol om effecten en mechanismen van voeding of specifieke voedingsstoffen te bestuderen. De overvloed aan data van nutrigenomics-studies kan waardevolle informatie opleveren voor allerlei aan voeding gerelateerde ziekten, zoals kanker, hart- en vaatziekten, suikerziekte (diabetes) en overgewicht (obesitas). De uitdaging voor de

komende tijd is om de analyse van data te vergemakkelijken en om meer ervaren te raken in de interpretatie van de grote hoeveelheden data. Hopelijk zal dit dan leiden tot belangrijke ontwikkelingen in de preventie van ziekte door voeding, zoals het ophelderen van mechanismen en het ontwikkelen van biomarkers voor vroege stadia van ziekte-ontwikkeling.

BEDANKT!

Tja, en dan is dat langverwachte moment toch echt aangebroken, tijd om iedereen die op welke manier dan ook heeft bijgedragen aan dit boekje te bedanken…

Natuurlijk moet ik dan beginnen met mijn begeleiders. Ben, vanaf ons eerste gesprek was je enthousiasme voor de nutrigenomics duidelijk en aanstekelijk. Dit enthousiasme en je eeuwigdurende optimisme heeft me altijd erg geholpen. Dat je het laatste jaar vaak op reis was, pakte gunstig uit, omdat je op reis vaak meer tijd had om hoofdstukken te lezen en sneller reageerde dan als je op TNO was. Bedankt voor alles, en je lijfspreuk 'maak je geen zorgen, alles komt goed' is ook nu weer uitgekomen! Ik vind het leuk dat we blijven samenwerken de komende tijd.
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My project was part of the EU-project 'Functional Food Ingredients Against Colorectal Cancer'. Ruud Woutersen, Anne Mensink, Ardy van Helvoort, Eric Caldenhoven, Ad Peynenburg, Jaap Keyer, Ole Vang and Renate Burgemeister, thank you for the cooperation and I always really enjoyed our meetings.

Centrum-AIO zijn, dat betekende in mijn geval 2 werkplekken (tox en TNO) en dus veel reizen tussen Wageningen en Zeist, met zip-diskettes, veel papieren (en dan vaak niet die papieren die ik echt nodig had…) en soms ook een witte piepschuim doos met monsters op droogijs (garantie op veel bekijks in de trein!). Maar naast twee bureaus betekende het ook twee keer kamergenoten, twee keer collega's en bijvoorbeeld twee keer labuitjes…..
Als ik na een paar dagen tox weer op TNO kwam of na een paar dagen TNO weer op tox kwam werd ik altijd weer enthousiast ontvangen door mijn kamergenoten. Wilbert, Ana, Marinus, Jelmer en Liesbeth bedankt daarvoor!
Wilbert en Jelmer, ik vind het leuk dat jullie mijn paranimfen willen zijn. De hele opkomst van de genomics op TNO, vanaf het prille begin met duizenden PCR reacties tot aan het einde met duizenden getallen in Excel, hebben we samen doorgemaakt, Wilbert. Het was een mooie tijd en hopelijk blijven we nog even kamergenoten. Jelmer, heel wat AIO-perikelen hebben we gedeeld in 1021. Het duurde even voordat het echt ons hok werd (weet je nog, die schoonmaak/opruiming??). Samen tijdens werktijd tennis op Wimbledon of Roland Garros volgen was top!

Zonder je mede-AIO's op tox en ook op TNO ben je nergens. Miriam (gelijktijdig proefschrift afmaken schept een band!), Wilbert, Anne en Ashwin op TNO en Yvonne (centrum-AIO van het eerste uur), Barry, Jelmer, Hester (Vichy!), Anne-Marie, Merijn (wanneer gaan we nou de 4-daagse lopen?), Marcel, Maaike, Suzanne, Pim, Wiratno, Vincent en Vincent in Wageningen, bedankt voor alle gezelligheid. Lieve tox-AIO's, ik zal de vrijdagmiddagborrels, de barbecues en etentjes, de zomers en het 'even binnenlopen en kletsen' missen! Ik hoop dat er nog veel promotiefeestjes volgen….
Natuurlijk zijn er nog meer collega's die ik wil noemen. Alle (oud-)toxers: Timo, Eric, Marlou, Arno, Daphne, Astrid, Magda, Juliette, Ineke, Mieke, Ivonne, Gerrit, Tinka, Irene, Gré, Annemarie, Bert, Hans, Ans, Laura en Marelle, mede dankzij jullie heb ik het altijd naar mijn zin gehad op tox.
En dan de TNO-ers. Een deel van het genomics-clubje is hierboven al genoemd. Michèle, bedankt voor alle praktische ondersteuning en de ontelbare RNA isolaties. Rob, vanaf het begin ben je, van een afstandje, bij mijn project betrokken geweest. Ik vind het leuk dat we nu weer samenwerken binnen NuGO. Cyrille, bedankt voor de samenwerking, het lezen van vele hoofdstukken en al je goede tips. Ook alle anderen van verklarende toxicologie / biomolecular sciences / physiological sciences, bedankt voor de gezelligheid bij de openhaard.
Natuurlijk kan ik de mensen van het arraylab niet vergeten. Vooral in de eerste jaren heb ik veel tijd in gebouw 9 doorgebracht! Frank en Ted (steun en toeverlaat in die tijd), Annemiek en Evelyn (voor de vrolijke noot in het lab), Mieke en Alie bedankt.
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Een aanzienlijke bijdrage aan dit boekje is geleverd door de studenten die bij mij, in Wageningen en/of in Zeist, een afstudeervak hebben gedaan. Yvonne, Renske, Paul, Joanne, Eva en Sylvie, het was leuk om met jullie samen te werken en bedankt voor al jullie werk.

Gelukkig is er ook nog leven naast het werk. Gea, het jaartje als huisgenoten (elke ochtend samen naar het station fietsen!) was en de weekendjes Brielle zijn altijd erg gezellig. Anouk, na samen afstuderen gaan we nu ook bijna tegelijk promoveren. Samen sporten, kletsen, lunchen en eten, bedankt voor je steun en alle gezelligheid. Cecile, nog 4 jaar langer samen in Wageningen heeft onze vriendschap echt versterkt, ook jij bedankt voor alles. Ik kom je snel opzoeken in Amerika! Lieve zonnetjes, lekker ontspannen met de jaarclub lukte altijd, tijdens etentjes, dagjes uit of vele kopjes thee. EPL-cluppie, bij jullie is het altijd vertrouwd en gezellig, ik hoop dat dat nog lang zo blijft.
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Naast twee dokters hebben we nu ook twee doctors in de familie!

CURRICULUM VITAE

Marjan Jolanda van Erk werd geboren op 2 april 1975 te Groningen. Op eenjarige leeftijd verhuisde ze met haar ouders naar Veldhoven. Na basisschool 'de Aanloop' vervolgde ze haar opleiding op het Eindhovens Protestants Lyceum te Eindhoven, waar ze in 1993 het gymnasium diploma behaalde. In datzelfde jaar begon ze met de studie Voeding en Gezondheid aan de Landbouwuniversiteit Wageningen (nu Wageningen Universiteit). Tijdens haar studie deed ze een afstudeervak bij de vakgroep Toxicologie onder begeleiding van Dr. Jac Aarts. Een tweede afstudeervak heeft ze uitgevoerd bij het RIVM te Bilthoven, onder begeleiding van Dr. Claudia Oomen. Als afsluiting heeft ze nog 5 maanden stage gelopen bij 'the Clinical Nutrition and Risk Factor Modification Centre' (University of Toronto) in Toronto, Canada, onder begeleiding van Professor David Jenkins. In september 1999 haalde ze haar doctoraal diploma met lof.

Vanaf oktober 1999 werd ze voor 4 maanden aangesteld bij de Wageningen Universiteit en werkte bij TNO Voeding aan het onderzoeksvoorstel voor haar promotieonderzoek. In februari 2000 begon ze met het promotieonderzoek, dat beschreven is in dit proefschrift. Het promotieonderzoek werd uitgevoerd bij Wageningen Universiteit, sectie Toxicologie en bij TNO Voeding, afdeling 'Physiological Sciences'. Sinds 1 april 2004 is ze werkzaam bij TNO Voeding, als postdoc nutrigenomics in 'the European Nutrigenomics Organization (NuGO)'.

LIST OF PUBLICATIONS

Full articles

Claudia M Oomen, Marjan J van Erk, Edith JM Feskens, Frans J Kok, Daan Kromhout. Arginine intake and risk of coronary heart disease mortality in elderly men. Arteriosclerosis, Thrombosis and Vascular Biology, 2000, 20: 2134-2139.

David JA Jenkins, Cyril WC Kendall, Edward Vidgen, Livia SA Augustin, Marjan van Erk, Anouk Geelen, Tina Parker, Dorothea Faulkner, Vladimir Vuksan, Robert G Josse, Lawrence A Leiter, Philip W Connelly. High-protein diets in hyperlipidemia: effect of wheat gluten on serum lipids, uric acid, and renal function. American Journal of Clinical Nutrition, 2001, 74: 57-63.

Marjan J van Erk, Paul Roepman, Ted R van der Lende, Rob H Stierum, Jac MMJG Aarts, Peter J van Bladeren, Ben van Ommen. Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. European Journal of Nutrition 2004 (online publication April 30).

Marjan J van Erk, Eva Teuling, Yvonne CM Staal, Sylvie Huybers, Peter J van Bladeren, Jac MMJG Aarts, Ben van Ommen. Time- and dose-dependent effects of curcumin on gene expression in human colon cancer cells. Journal of Carcinogenesis, 2004, 3:8.

MVW Wijnands, MJ van Erk, RP Doornbos, CAM Krul, R Woutersen. Predictive value of ACF for the occurrence of colorectal tumours; potential of gene expression profiling in tumours. Food and Chemical Toxicology 2004, in press.

Marjan J van Erk and Ben van Ommen. Functional genomics and gastrointestinal health promotion. In: Nutrigenomics and Proteomics in Health Promotion and Disease Prevention. To appear in February 2005.

Marjan J van Erk, Cyrille AM Krul, Eric Caldenhoven, Rob H Stierum, Ruud A Woutersen, Ben van Ommen. Development of an in vitro screening system for potential cancer-preventive compounds based on gene expression profiling of cell lines and of human colon biopsies. Submitted for publication.

Marjan J van Erk, Cyrille AM Krul, Marcel VW Wijnands, Ruud A Woutersen, Ben van Ommen. Gene expression profiling of effects of plant compounds in relation to colon carcinogenesis. Submitted for publication.

Marjan J van Erk, Cyrille AM Krul, Eric Caldenhoven, Rob H Stierum, Ruud A Woutersen, Jac MMJG Aarts, Ben van Ommen. Screening for potential cancer-preventive effects of quercetin, curcumin and resveratrol by gene expression profiling in colon cancer cell lines. In preparation.

Abstracts

Marjan van Erk, Yvonne Dommels, Frank Schuren, Ted van der Lende, Karin Schütze, Rob Stierum en Ben van Ommen. Colon carcinogenesis studied by laser microdissection and DNA microarrays. EuroConference 'Microdissection and its downstream tools', Bonn-Königswinter, Germany (Sept 15-17, 2000).

Marjan van Erk, Rob Stierum, Ted van der Lende, Ben van Ommen. The influence of quercetin on multiple gene expression in Caco-2 cells. Annual Meeting of the American Association for Cancer Research, New Orleans, USA (March 24-28, 2001).

Marjan van Erk, Jac Aarts, Peter van Bladeren, Ben van Ommen. Using microarrays to study nutrient-gene interaction in the colon. ESFS-JSPS Frontier Science Meeting for Young Researchers: Functional Genomics – from the bench to bioinformatics. San Feliu de Guixols, Spain (Oct 25-31, 2003).

Marjan van Erk, Eva Teuling, Yvonne Staal, Sylvie Huybers, Peter van Bladeren, Jac Aarts, Ben van Ommen. Effect of curcumin on gene expression in human colon cancer cells. First International Conference on Polyphenols and Health, Vichy, France (Nov 18-21, 2003).

Marjan J van Erk, Cyrille A.M. Krul, Eric Caldenhoven, Rob H. Stierum, Ruud Woutersen, Ben van Ommen. Functional Food Ingredients against Colorectal Cancer. Annual Meeting of the American Association for Cancer Research, Orlando, USA (March 27-31, 2004).

Training and supervision plan

Overview of conferences and courses attended during PhD

Workshop on microdissection (Germany)	2000
Conference Microdissection and it downstream tools (Germany)	2000
Cancer genetics course (Italy)	2000
Cell signaling symposium	2000
Cursus Afstudeervak begeleiden en organiseren	2000
Bioinformatics meeting	2001
Annual meeting of the American Association for Cancer Research (USA)	2001
Start symposium of Centre for Human Nutrigenomics	2001
Course From nutrigenomics to healthy foods	2001
EU Workshop Applications of molecular biology techniques in nutritional research in Europe (England)	2001
Genomics Momentum	2001
Course Writing and presenting a scientific paper	2001
Theme days Centre for Human Nutrigenomics	2002
Userday i-Cycler	2002
Expression profiling course	2002
Workshop Statistical aspects of microarray data (Denmark)	2003
Discussion symposium nutrigenomics	2003
Symposium Transcriptional and other regulatory mechanisms of lipid metabolism (Germany)	2003
ESFS-JSPS Frontier science meeting for young researchers: Functional genomics – from the bench to bioinformatics (Spain)	2003
First international conference on polyphenols and health (France)	2003
Annual meeting of the American Association for Cancer Research (USA)	2004

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