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## Time- and dose-dependent effects of curcumin on gene expression in human colon cancer cells

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### Abstract

**Background:** 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.

## Background

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 diet 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 leukaemia 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 faeces [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 components. 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 5 A medium with L-glutamine (Gibco BRL Life Technologies) with 10% FCS. Caco-2 cells (ATCC, Rockville, USA) were grown in DMEM with 25 mM 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°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Curcumin (Sigma, St. Louis, MO, USA) was dissolved in DMSO. At 70% confluence HT29 cells and Caco-2 cells were exposed to 30 μmol/L (referred to as low concentration) or 100 μmol/L curcumin (referred to as high concentration) for 3 or 6 hours. In addition, HT29 cells were exposed to 25 μmol/L (referred to as low concentration) or 100 μmol/L 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  $3 \times 10^5$  cells in 25 cm<sup>2</sup> 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  $500 \times g$  at 4°C 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 analysed 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 labelling and hybridisation 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 1× 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 CCG 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'-CTGCAAGGCCGATTAAGTTGGTAAC-3') and reverse (5'-GTGAGCGGATAACAATTTACACAGGAAACAGC-3') primers. The primers contained a 5'-C6-aminolinker (Iso-gen Bioscience, Maarsse, 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  $3 \times$  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.

**Labelling and hybridisation**

Total RNA (25 µg) was labelled using CyScribe first-strand cDNA labelling 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°C, RNA was hydrolysed by adding NaOH. After neutralisation, free nucleotides

were removed from the solution using AutoSeq G50 columns (Amersham Biosciences, Freiburg, Germany).

Each sample was labelled twice, once with Cy3 and once with Cy5. cDNA from cells exposed to curcumin (treated sample) was hybridised to the microarray in competition with cDNA from cells exposed to DMSO only (untreated sample), while one of the samples was labelled with Cy3 and the other with Cy5. Hybridisations 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 hybridisation mix was dissolved in 30 µl Easyhyb hybridisation buffer (Roche Diagnostics, Mannheim, Germany), denatured for 1.5 min at 100°C and incubated for 30 minutes at 42°C.

Before adding the hybridisation mix to the slides, slides were prehybridised in prehybridisation buffer (5 × SSC, 0.1 % SDS and 10 mg/ml bovine serum albumin) for 45 minutes at 42°C, washed in milliQ water, washed with isopropanol and dried.

After pipetting the hybridisation mix on the slides, the slides were covered with a plastic coverslip and hybridised overnight in a slide incubation chamber (Corning, Life Sciences, Schiphol, the Netherlands) submerged in a 42°C waterbath. After hybridisation, slides were washed by submersion and agitation in 0.5 × SSC with 0.2% SDS and in 0.5 × SSC. Then, slides were firmly shaken in 0.2 × 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 Imagen 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 Imagen 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), normalised per slide using an intensity-dependent method (Lowess) [30] and scaled. Data were transferred to Microsoft Excel 97 (Microsoft Corporation, USA). Expression ratios of duplo (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 timepoint were excluded from further analysis and when looking for early or late response genes only genes that did not show dye bias were included.

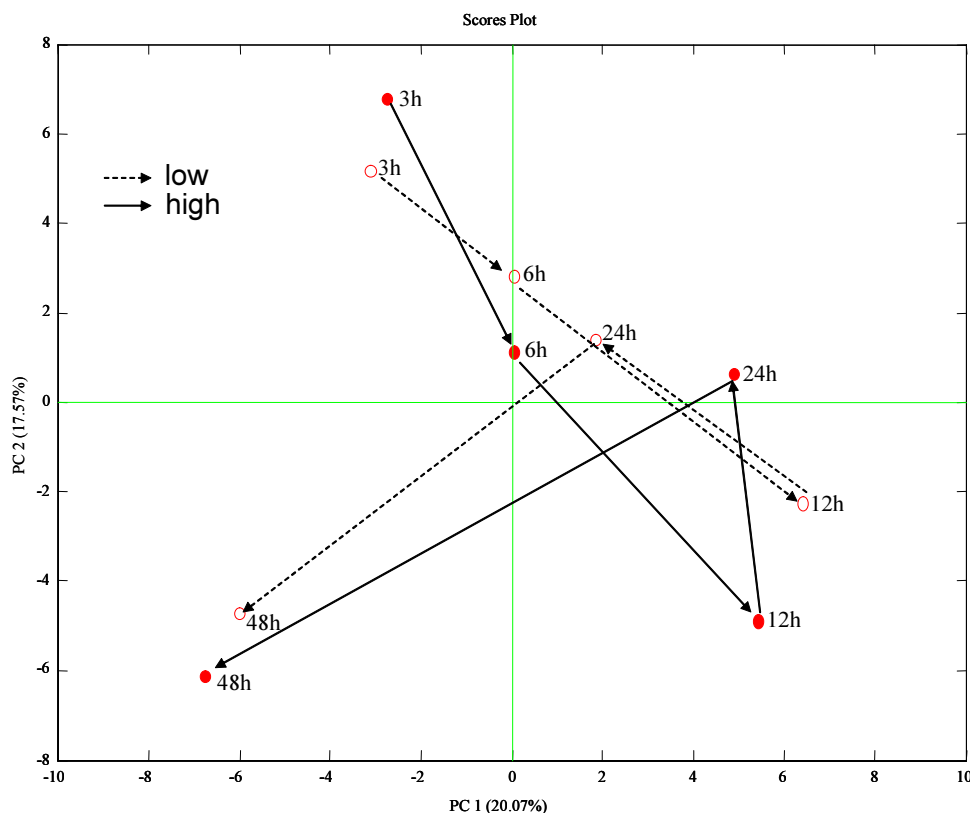
Since it is impossible to report results for all genes on the array for all time points, we chose to look at genes with a relatively large change in expression over time. Among these genes we identified early response genes (which were more than 1.5 fold induced or repressed after 3 or 6 hours of exposure) 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 1) nicely visualizes the time-dependent changes in the gene expression patterns. Interestingly, the time-dependent shift of the overall gene expression



### Figure 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.

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.

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 1 (low concentration) and table 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 1 and 2). Upregulated signal transduction genes included STAT3 and STAT5b (Table 1) and some genes of the MAPK signal transduction pathway (MAP3K10, MAP4K2; table 1 and 2). Some other MAPK signal transduction genes were downregulated by the low curcumin concentration (Table 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 1) and integrin genes (Table 2). Several genes involved in xenobiotic metabolism were downregulated after short-term exposure to the high concentration of curcumin, namely GSTT2, GSTM4, CYP1B1 (Table 2). Expression of GCLC, involved in glutathione synthesis, was upregulated after 6 hours (Table 2).

Also, genes involved in transcription regulation were induced or repressed by short-term exposure to curcumin.

**Table 1: Early response genes in HT29 cells after exposure to low concentration of curcumin (30 µmol/L).**

| Accession number | Gene Name   | Gene Symbol | Fold change<br>(treated/control) |                 | Involved in                                  |
|------------------|---|-------------|----------------------------------|-----------------|--|
|                  |   |             | 3 h                              | 6 h             |  |
| 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 <sup>1</sup> | 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 homolog 2 (yeast)  | SMT3H2      | -1.77                            | -1.57           | Nuclear transport                            |
| 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                          |

<sup>1</sup> NA: not available

Among these were transcription factors such as activating transcription factor 4 (ATF4) and early growth response 1 (EGR1) (Table 2). One of the target genes regulated by ATF4 is asparagine synthetase (ASNS) [31]. Figure 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

**Table 2: Early response genes in HT29 cells after exposure to high concentration of curcumin (100 µmol/L).**

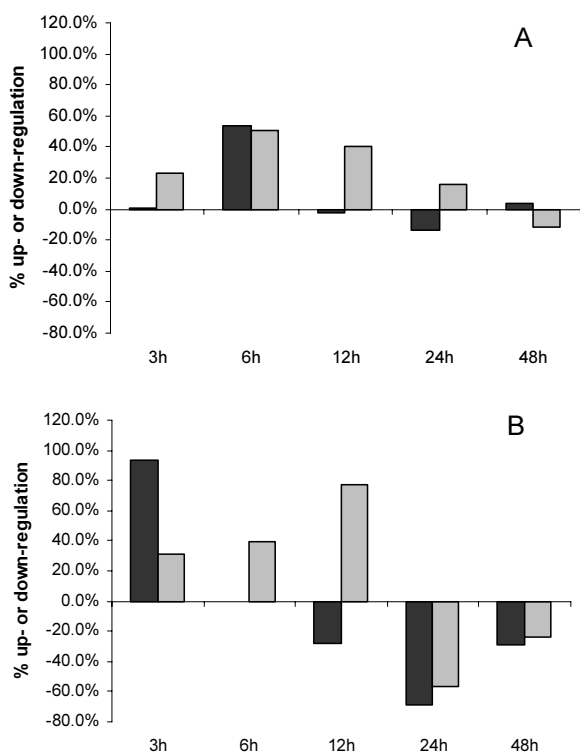
| Accession number | Gene Name  | Gene Symbol | Fold change<br>(treated/control) |                 | Involved in                       |
|------------------|--|-------------|----------------------------------|-----------------|-----------------------------------|
|                  |  |             | 3 h                              | 6 h             |                                   |
| 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 <sup>1</sup> | 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, 30 kDa   | 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  |
| 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  | ETRI01      | 1.54                             | 1.10            | Unknown                           |
| AA425687         | DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 1   | DDX1        | -1.56                            | NA              | Unknown                           |

<sup>1</sup> NA: not available

induced by the low curcumin concentration (Figure 3A). 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 ( $23.0 \pm 2.73$  when normalised to beta-actin and  $30.5 \pm 2.96$  when normalised 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).

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 2). At the same time point, expression of cyclin-dependent kinase inhibitor p16INK4 (CDKN2A)



**Figure 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.

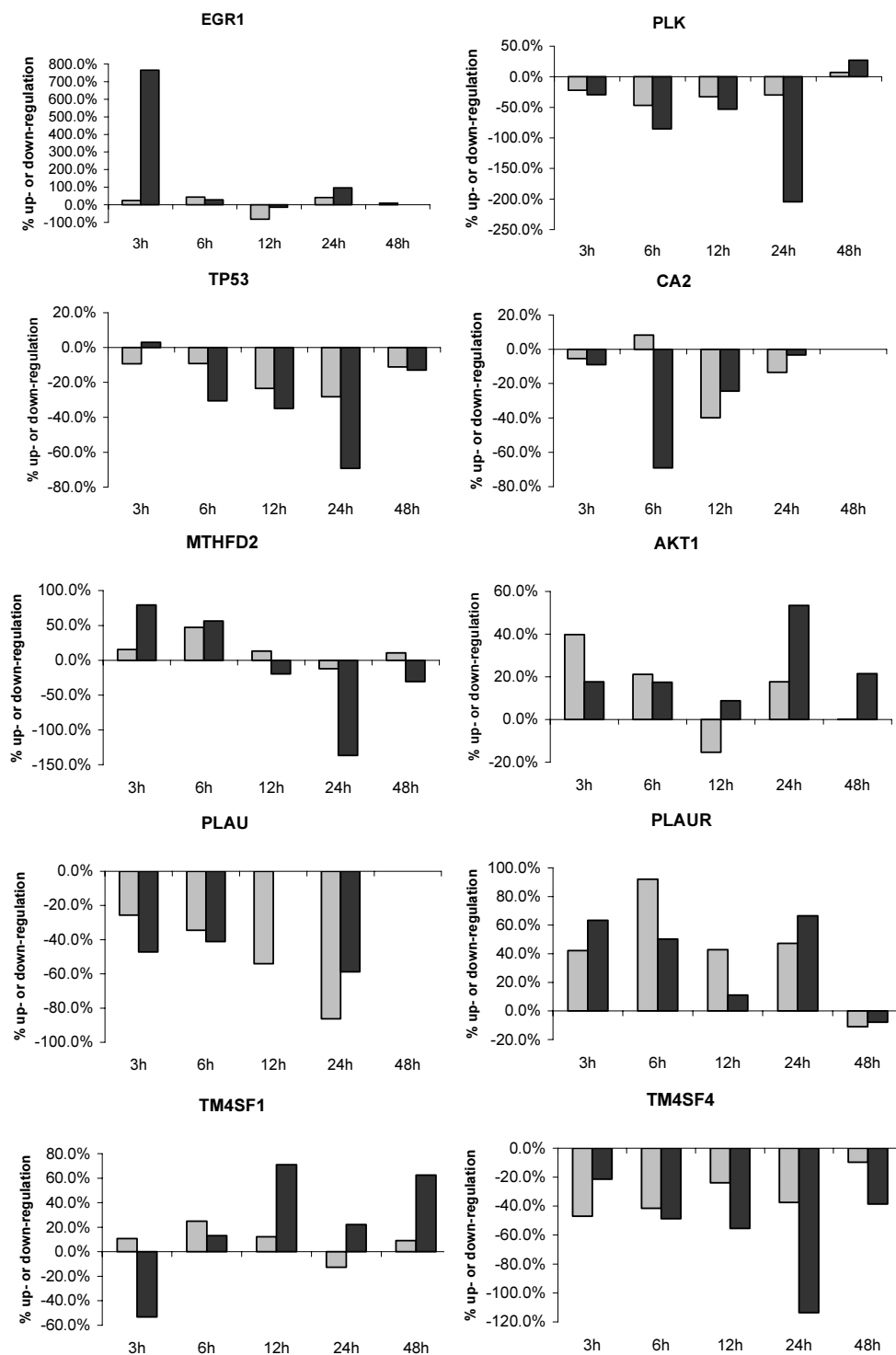
was upregulated (Table 2). Expression of cell growth related genes PCNA and IGFBP7 was downregulated by the high concentration of curcumin (Table 2). Also after exposure to the low curcumin concentration some cell cycle genes were downregulated, like BUB1B (a mitotic checkpoint gene), STK6, STK12 and cyclin G1 (CCNG1). Upregulated cell cycle genes included CDK10, CDC5L and cyclin E1 (CCNE1), but also cell cycle inhibitor p16INK4 (CDKN2A) (Table 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 (fig. 3B). 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 12 h and 24

h 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. 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). In addition, expression of urokinase-type plasminogen activator receptor (PLAUR) was upregulated by curcumin (Figure 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 and 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). 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) 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), 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].

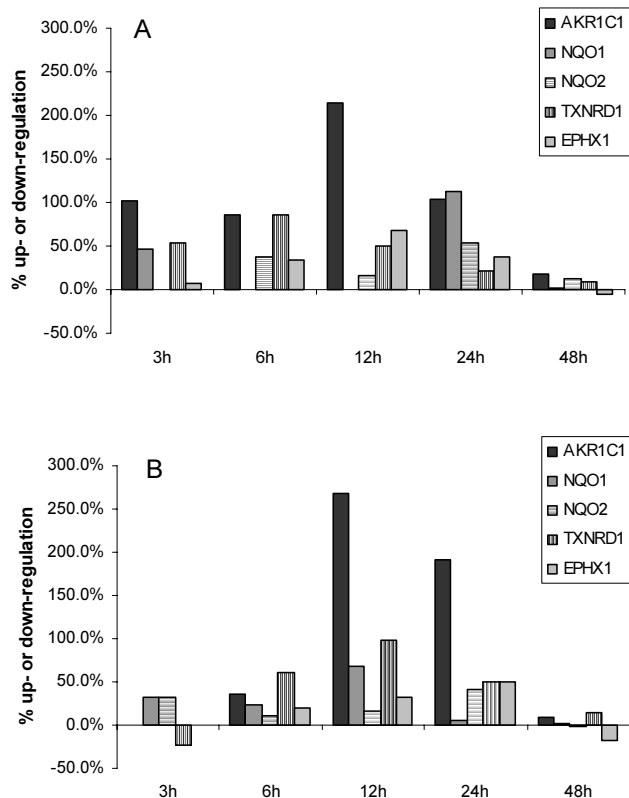
Clustering methods were used to identify groups of genes with similar expression profile across the different time points. Several functional groups of genes with a similar





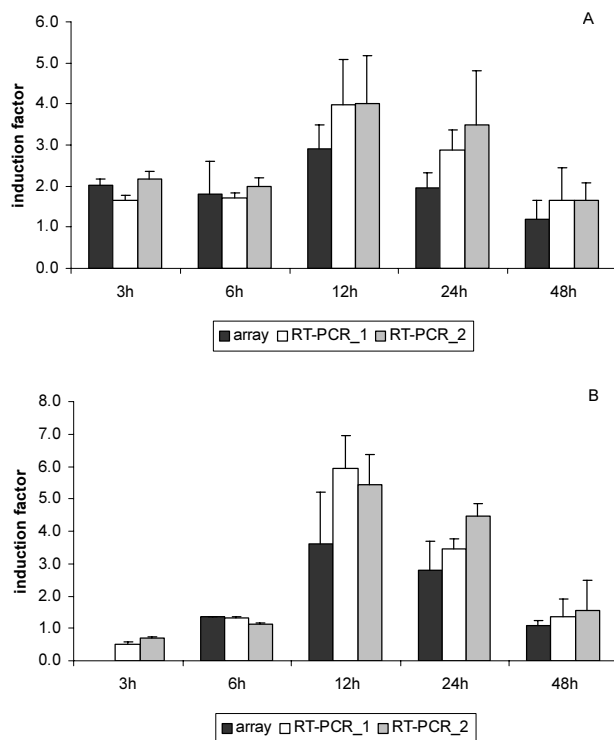
**Figure 3**

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.



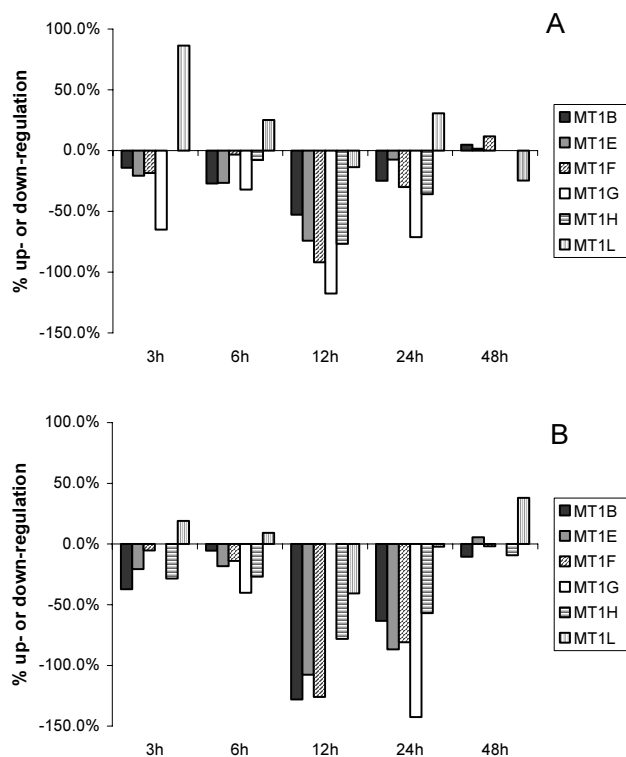
**Figure 4**  
Response of genes involved in phase II metabolism to exposure to curcumin; A: low concentration, B: high concentration. AKR1C1: aldo-keto reductase family I member C1; NQO1: NAD(P)H dehydrogenase, quinone 1; NQO2: NAD(P)H dehydrogenase, quinone 2; TXNRD1: thioredoxin reductase I; EPHX1: epoxide hydrolase I.

expression profile were found. Figure 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 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 oxi-



**Figure 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  $\pm$  standard deviation).

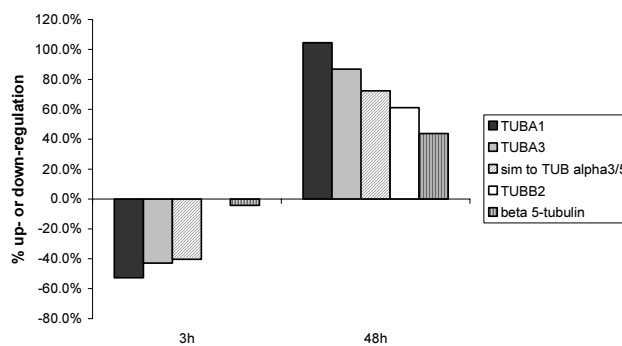
doreductase 1 (NQO1) and heavy subunit of glutamate-cysteine ligase (GCLC) in a colon cancer cell line [43]. These genes were also upregulated by curcumin in this study. In addition, NQO2 and epoxide hydrolase 1 (EPHX1) were also upregulated (Figure 4). Several other redox-sensitive genes were upregulated after short-term exposure to the high concentration of curcumin, like AREG, ATF4, EGR1, FGFR1 (Table 2) [44]. However, not all phase-II genes were upregulated by curcumin. In this study several GSTs were downregulated, e.g. GSTT2 and GSTM4 (table 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).



**Figure 6**  
Response of metallothionein (MT) genes IB-IL to exposure to curcumin; A: low concentration, B: high concentration.

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 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).

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 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 1). In human colon cancer cells metallothionein was found to be a possible proliferation



**Figure 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.

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 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].

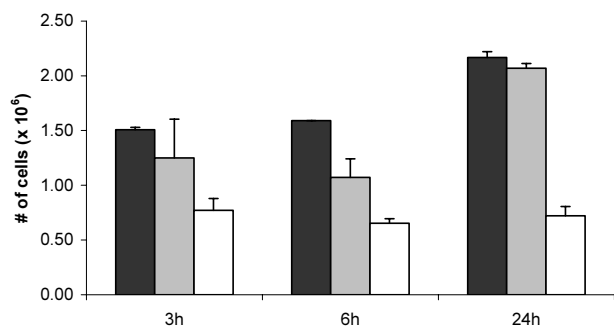
Several proteasome genes were upregulated by the high concentration of curcumin after 12 or 24 hours: PSMA1, PSMA7, PSMB2 after 12 hours and PSMB6, PSMC4, PSMD2 after 24 hours (data not shown).

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). CYP1A1 expression did not change in HT29 cells in response to curcumin, however CYP1B1 expression was downregulated by the high concentration of curcumin (Table 2). Some genes showed a similar response in Caco-2 cells as in HT29 cells, for example EGR1 and AKR1C1. However, upregulation

**Table 3: Early response genes in Caco-2 cells after exposure to curcumin.**

| Accession number | Gene Name   | Gene Symbol | Fold change (treated/control) |                 |            |       | Involved in           |
|------------------|---|-------------|-------------------------------|-----------------|------------|-------|-----------------------|
|                  |   |             | 30 µmol/L                     |                 | 100 µmol/L |       |                       |
|                  |   |             | 3 h                           | 6 h             | 3 h        | 6 h   |                       |
| 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 <sup>1</sup> | -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, 30 kDa                              | 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               |

<sup>1</sup> NA: not available



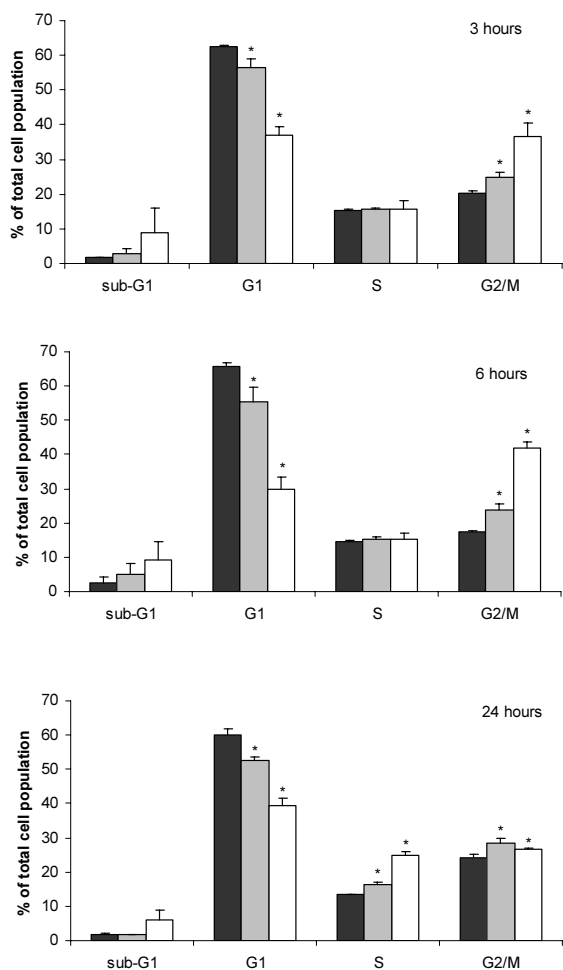
**Figure 8**  
Number of HT29 cells after exposure to curcumin at different time points. Black bars: untreated cells, grey bars: low concentration, white bars: high concentration. Values are mean ± standard deviation.

in Caco-2 cells (Table 3) was not as strong as in HT29 cells (Figure 4 and 3). Expression of TLOC1 and POLR1C was downregulated in Caco-2 cells (Table 3) as well as in HT29 cells after short-term exposure (Table 1 and 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).

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 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 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 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 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

**Figure 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  $\pm$  standard deviation (n = 3 or 4). \*: significantly different from untreated cells, P < 0.05

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).

## 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* sit-

uation. Based on almost complete faecal 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  $\mu\text{mol/L}$  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  $\mu\text{mol/L}$ . 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  $\mu\text{mol/L}$  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 leukaemia 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 signalling 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  $\mu$ M) were measured at time points between 30 minutes and 48 hours. Of the 6253 genes analysed 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 (unpublished results). 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.

## Conclusions

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.

## Authors' contributions

ME, ET, YS and SH carried out the cell culture, RNA isolation, microarray studies and flow cytometry studies. ME carried out the real-time PCR, the microarray data analysis and drafted the manuscript. PB, JA and BO participated in design of the study and of the manuscript. All authors read and approved the manuscript.

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