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Expression Profiles of Apoptotic Genes Induced by Curcumin in Human Breast Cancer and Mammary Epithelial Cell Lines

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Abstract. *Curcumin (diferuloyl methane), the yellow-colored dietary pigment from the rhizomes of turmeric, has been recognized as a chemopreventive agent because of its antitumor, antioxidant and antiproliferative effects. The cytotoxic, apoptotic and gene regulatory effects of both turmeric and curcumin were investigated in the MCF-7 human breast cancer carcinoma cell line and compared with the effects in MCF-10A human mammary epithelial cells. MCF-7 cells were more sensitive to turmeric and curcumin than MCF-10A cells. MCF-10A cells retained comparatively less curcumin in the medium than MCF-7 cells after 24 h, thereby reducing the cytotoxic effect. Curcumin induced a significantly higher percentage of apoptosis in MCF-7 than MCF-10A cells at all doses. Microarray hybridization of Clonetech apoptotic arrays with labeled first-strand probes of total RNA was performed to identify and characterize the genes regulated by curcumin in tumor cells. Of the 214 apoptosis-associated genes in the array, the expression of 104 genes was altered by curcumin treatment. The gene expression was altered up to14-fold levels in MCF-7 as compared to only up to 1.5-fold in the MCF-10A cell line by curcumin. Curcumin up-regulated (>3 fold) 22 genes and down-regulated (<3-fold) 17 genes at both 25 μg/ml and 50 μg/ml doses in the MCF-7 cell line. The up-regulated genes include HIAP1, CRAF1, TRAF6, CASP1, CASP2, CASP3, CASP4, HPRT, GADD45, MCL-1, NIP1, BCL2L2, TRAP3, GSTP1, DAXX, PIG11, UBC, PIG3, PCNA, CDC10, JNK1 and RBP2. The down-regulated genes were TRAIL, TNFR, AP13, IGFBP3, SARP3, PKB, IGFBP, CASP7, CASP9, TNFSF6, TRICK2A, CAS, TRAIL-R2, RATS1, hTRIP, TNFb and TNFRSF5. While a dose-dependent gene expression change was noticed in some genes, opposite regulatory effects were induced by different curcumin doses in three apoptotic genes. These results suggest that curcumin induces apoptosis in breast cancer cells by regulation of multiple signaling pathways, indicating its potential use for prevention and treatment of cancer.*

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Curcumin, a well-known chemopreventive agent from turmeric, inhibits a number of cellular processes involved in carcinogenesis and tumor growth. It regulates an array of cellular processes such as inhibition of lipid peroxidation, nitric oxide synthetase activity, epidermal growth factor (EGF) receptor kinase C activity, NF-Î B activity, protein kinase C activity and production of reactive oxygen species (1-4). This chemopreventive agent is also reported to inhibit the expression of several proto-oncogenes and cell proliferation genes in tumor cells (5, 6). It is a potent inhibitor of mutagenesis and chemically-induced carcinogenesis in animal tumor models (7, 8). Curcumin also inhibits type I human immunodeficiency virus (HIV-1) replication by down-regulating the long terminal repeat (LTR)-directed *p24* gene expression (9). We have previously reported that curcumin induces a high percentage of apoptosis in human breast cancer cells by regulating the expression of genes associated with programmed cell death (10). It down-regulates *Ki67, PCNA* and *p53* mRNAs in breast cancer cell lines that may underlie its chemopreventive action. Recently, we showed that curcumin inhibits human telomerase activity by down-regulating *hTERT* gene expression in breast cancer cells and that this down-regulation is not through the c-myc pathway (11).

The effectiveness of any chemotherapeutic or chemopreventive agent depends on its cytotoxic effect on tumor cells in relation to normal cells. The normal as well as tumor cells respond to these agents differently because of their inherent gene expression patterns. Most of the previous studies have restricted their focus to a small group of candidate genes to determine the effectiveness of these agents. Therefore, they have not taken into account the

broader set of genes involved in the cell death process. Microarray hybridization provides a unique opportunity to study the extent and degree of gene expression changes induced by cytotoxic agents as a consequence of therapy. Microarray studies using RNAs of colon carcinoma cells treated with curcumin for varying time-periods have been performed to identify genetic reprogramming in pathways of colonic cell maturation (12). Even though individual gene expression data are available for curcumin, global gene expression changes induced by this agent relative to normal cells have not been studied systematically to date. In this investigation the changes in the expression of various apoptotic genes induced by curcumin in human breast cancer and mammary epithelial cell lines were analyzed in relation to their cytotoxicity levels.

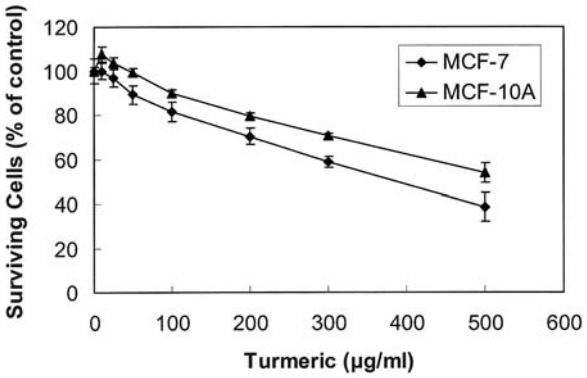

Figure 1. *Turmeric cytotoxicity of human breast cancer and mammary epithelial cells. Cells were incubated with varying doses turmeric for 24 h and cytotoxicity estimated by MTT assay. The mean percentage of surviving cells (n=3) and standard deviation estimates were plotted against turmeric concentrations.*

Materials and Methods

Cell lines. Human breast adenocarcinoma (MCF-7) and mammary epithelial (MCF-10A) cell lines were grown in DMEM medium supplemented with 10% FBS and antibiotics (10).

Turmeric and curcumin. Turmeric powder was purchased from Garry & Sun (Reno, NV, USA) and curcumin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The major constituents of turmeric are curcumin, curcuminoids and curcuma oil (particularly turmerone) (13). Commercial curcumin is reported to contain curcumin (77%), demethoxycurcumin (17%) and bisdemethoxy-curcumin (3%), together referred to as curcuminoids (14).

Cytotoxicity assay. Curcumin cytotoxicity was determined by cell proliferation (MTT assay) kit I (Roche Biochemicals, Indianapolis, IN, USA). Stock solution of curcumin was initially prepared in ethanol at 10 mg/ml concentration for cytotoxicity assay. MCF-7 and MCF-10A cells were treated with 0, 10, 25, 50, 75, 100, 150 and 200 µg/ml of turmeric or curcumin in DMEM medium in a CO_2 incubator for 24 h before the cytotoxicity assay (10). Each experiment was repeated three times with three replications at every concentration.

Curcumin retention. In our cytotoxicity experiments, an increased retention of yellow color (curcumin) was observed in the wells having cancer cells (MCF-7) compared to the mammary epithelial cells (MCF-10A) after 24 h. Hence, the curcumin concentrations in the medium as well as within the cells were estimated using a spectrophotometer. After 24 h of curcumin-treatment of cells, about 2 ml of medium was transferred from the wells to cuvettes and the absorbance was measured at 550 nm in a Beckman Spectrophotometer (15, 16). To determine the intracellular curcumin, the cells were washed three times with PBS and curcumin was extracted in 2 ml of alcohol-acid solution containing 50% ethanol and 0.3 M HCl. Statistical analysis of curcumin values for MCF-7 and MCF-10A cells were performed with Student's *t*-test.

Apoptosis assay. Curcumin-induced apoptosis was analyzed using the Annexin V-EGFP Apoptosis Detection kit from Medical & Biological Laboratories Co., Ltd. (Woburn, MA, USA). Tumor cells ($0.5x10^6$) were centrifuged in Eppendorf tubes and resuspended in 200 µl of 1x Binding Buffer. Annexin V-EGFP (1 µl) and propidium iodide (PI, 1 µl) were added to the tubes. The cells were incubated at room temperature for 5 min in the dark and analyzed in a Beckman-Coulter Elite flow cytometer. Annexin V-EGFP binding was detected using a FITC signal detector (FL1) and PI staining by the phycoerythrin emission signal detector (FL2). Percentages of FITC/PI-positive cells (apoptosis) were calculated from flow histograms (17, 18).

Microarray hybridization of apoptotic array

a. Extraction and purification of RNA using DNAse: Total RNA was extracted from cells using Perfect RNA kit (Eppendorf Scientific Inc., Westbury, NY, USA). DNA contamination in the RNA sample was removed by treating total RNA (10 µg) with 10 units of DNAse in 1X reaction buffer (Clonetech Laboratories, Inc., Palo Alto, CA, USA).

b. Preparation of first-strand DNA using purified RNA: First-strand cDNA was synthesized from 1 µg total RNA using the SMART II PCR cDNA synthesis kit (Clonetech Laboratories, Inc.).

c.PCR amplification of cDNA: cDNA was amplified using the Advantage DNA PCR kit (Clonetech Laboratories). In this process, PCR products were labeled with digoxigenin dUTP (Roche Biochemicals). PCR cycling conditions were 95˚C for 5 sec, 65˚C for 5 sec and 68˚C for 6 min for a total of 15 cycles (19, 20).

d. Hybridization of labeled cDNAs to apoptotic membrane array: Membrane arrays were prehybridized with a hybridization solution containing 5 µl of human cot-DNA and 5 µl of poly dA in 10 ml of Dig-Easy (Roche Biochemicals), for 4 h at 42˚C in a hybridization oven. The prehybridization solution was replaced with hybridization solution containing 50 µl cDNA probe and 12.5 µl of cot-DNA in 5 ml Dig-Easy hybridization solution (Roche Biochemicals). Hybridization bottles were incubated overnight at 42˚C in the rotating hybridization oven (20, 21).

e. Washing of microarrays and detection of hybridization signals: The membranes were washed with 2x SSC (sodium chloride: sodium

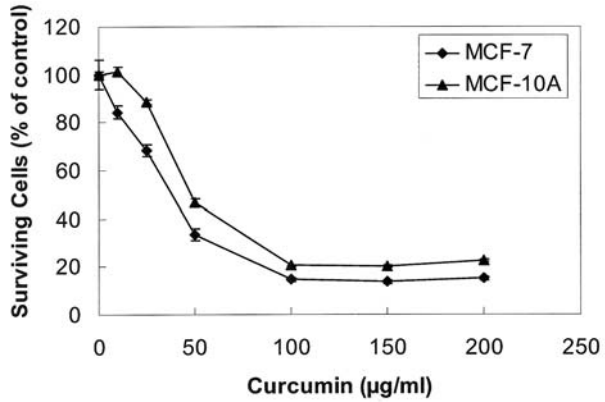

Figure 2. *Curcumin cytotoxicity of human breast cancer and mammary epithelial cells determined by MTT assay after incubating cells for 24 h. The mean percentage of surviving cells (n=3) and standard deviation estimates were plotted against cucumin concentrations.*

citrate buffer) and 0.1% SDS (Sodium dodecyl sulfate) for 3 h with frequent changes of buffer. Hybridization signals were detected with anti-digoxigenin/alkaline phosphatase conjugate and CDP-Star substrate. The membranes were exposed to Lumifilm (Roche Biochemicals) for 10 min after incubating them with the substrate for 5 min (19, 20).

f. Analysis of microarray: Four microarray membranes were processed for each curcumin concentration. The microarray autoradiographs were scanned and the images were analyzed with Atlas Image 2.0 (Clonetech Laboratories) and Expression Profile data CLUSTering and analysis (EPCLUST) programs. Briefly, the images were acquired and converted to Tagged Image File (.tif) format using a flatbed scanner. These array images were initially analyzed using Atlas Image software that subtracted background and normalized intensity on the basis of the lowest negative control as 0 and the highest positive control as 100. The data were copied into Microsoft Excel for further analysis. These array image files were then analyzed using the EPCLUST program accessible through the internet.

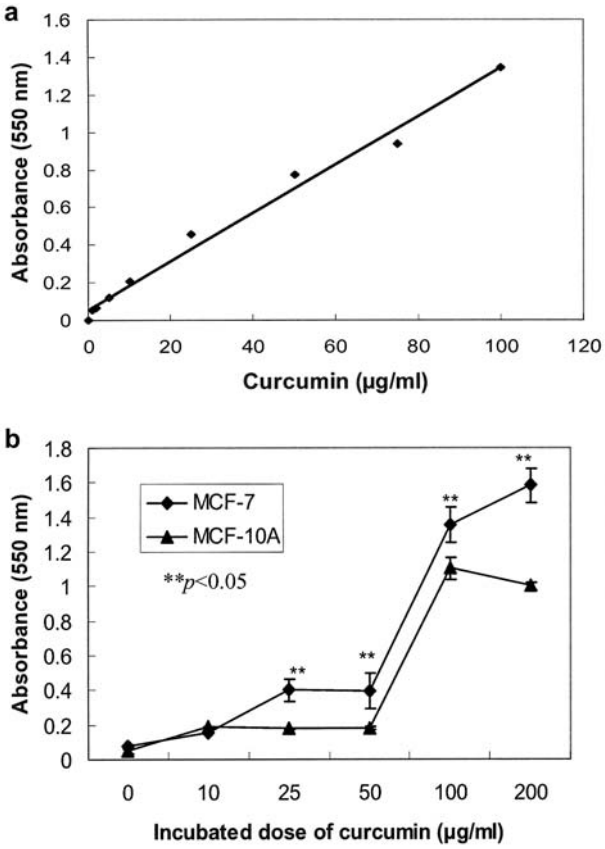

Figure 3. *a: Standard curve of curcumin showing the linearity of absorbance values against curcumin concentrations. Curcumin was diluted in cell culture medium and the absorbance recorded in a Beckman spectrometer at a wavelength of 550 nm. The absorbance values were plotted against curcumin concentrations using the Excel program. b: The retention of curcumin in the medium of breast cancer and mammary epithelial cells after 24 h of treatment. Cells were incubated with varying doses of curcumin for 24 h and the curcumin concentration in the medium analyzed in a spectrophotometer at 550 nm wavelength. The mean and standard deviation values were plotted against incubated curcumin doses.*

Results

Turmeric and curcumin cytotoxicity: Figures 1 and 2 show turmeric and curcumin cytotoxicity values of the MCF-7 and MCF-10A cell lines. Both turmeric and curcumin induced cell death, although the active ingredient curcumin was more cytotoxic than turmeric. Human breast carcinoma cells were more sensitive to curcumin and turmeric than mammary epithelial cells. Although 405 µg/ml turmeric induced 50% cell death in MCF-7 cells, MCF-10A cells showed less than 50% cell death even at 500 µg/ml turmeric. The curcumin IC_{50} value of MCF-7 was 29 µg/ml compared to 55 µg/ml for MCF-10A cells in a 24-h incubation period.

Curcumin retention. A change in color of the medium was observed after 24 h of curcumin treatment of the MCF-7 and MCF-10A cells. Figure 3a shows the standard curve of curcumin illustrating the linearity of relationship between curcumin concentrations and absorbance values. The curcumin concentration in the medium was measured in a spectrophotometer at 550 nm and is presented in Figure 3b. MCF-10A cells retained less curcumin in the medium than MCF-7 cells, especially above the 10 µg/ml dose. The intracellular curcumin concentrations after 24 h of treatment were not significantly different between MCF-7 and MCF-10A cells. MCF-7 and MCF-10A cells had an intracellular concentration of 1 µg/ml and 10 µg/ml curcumin at incubated doses of 50 µg/ml and 100 µg/ml, respectively, after 24 h.

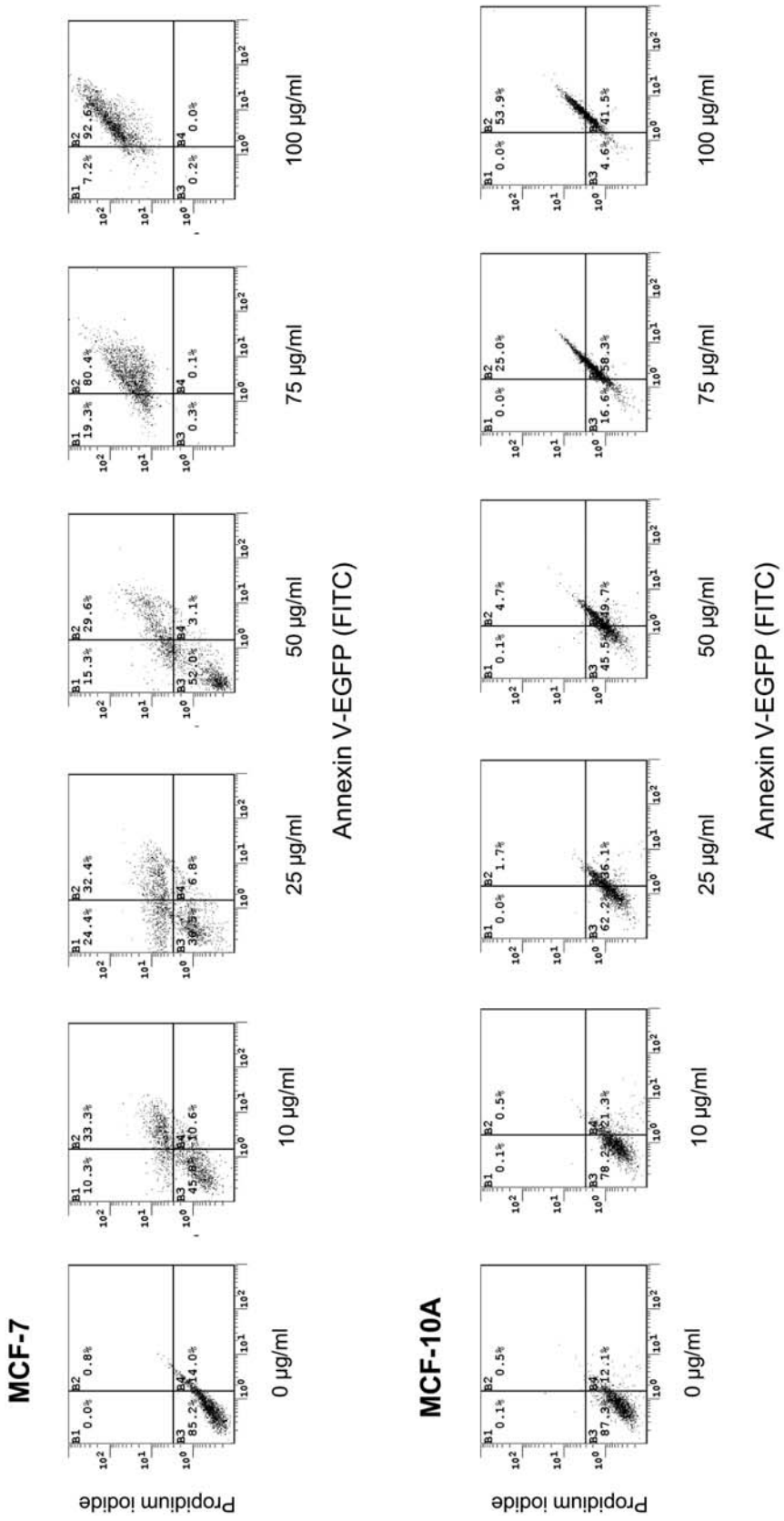

Figure 4. *Flow cytometric analysis of curcumin-induced apoptosis analyzed using the Annexin-V-EGFP assay kit. The cells falling in the upper-right square (B2) in each scatter diagram are considered as apoptotic cells.*

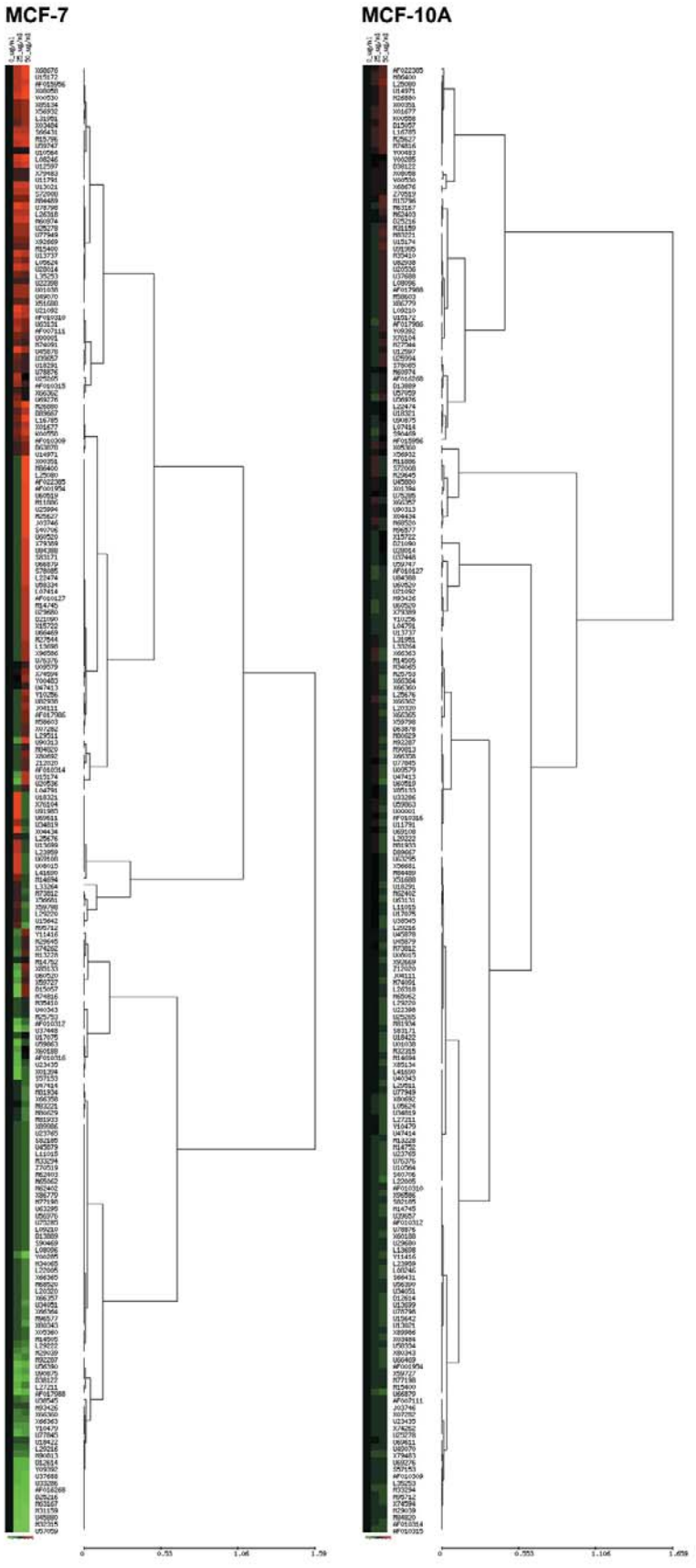

Figure 5. *Recruitment of changes in gene expression induced by curcumin in MCF-7 and MCF-10A cells. Tree-view and cluster analysis were performed with the EPCLUST program to group and display genes that were induced (red), suppressed (green) or unchanged (black) in expression after 24 h of curcumin treatment at 0, 25 and 50 μg/ml doses. The changes (red or green) in gene expression profiles were more evident in MCF-7 than in MCF-10A cells. A dose-dependent variation in gene expression pattern was also clear in the cluster diagram for certain genes.*

Table I. *Genes up-regulated at both curcumin doses (>3-fold).*

Gene bank number	Protein/gene name	Gene symbol	Curcumin (25 μg/ml)	Curcumin (50 μg/ml)
S66431	retinoblastoma-binding protein	*RBP2*	3.54	3.91
L26318	c-jun N-terminal kinase 1	*JNK1*	3.89	3.57
S72008	CDC10 protein homolog	*CDC10*	3.91	3.99
M15796	proliferating cyclic nuclear antigen	*PCNA*	4.89	5.58
AF010310	p53-inducible protein 6	*PIG 6*	6.97	4.91
AF010309	p53-inducible protein 3	*PIG3*	7.18	9.46
M26880	ubiquitin	*UBC*	9.00	14.02
AF010315	p53-inducible protein 11	*PIG11*	9.29	6.75
AF015956	death-associated protein 6	*DAXX*	9.71	11.45
X08058	glutathione S-transferase pi	*GSTP1*	10.23	13.02
U12597	tumor necrosis factor type 2 receptor-associated protein	*TRAP3*	10.28	11.49
U13699	caspase-1	*CASP1*	10.29	4.52
U59747	apoptosis regulator bclw	*BCL2L2*	10.44	11.24
U13021	caspase-2 precursor	*CASP2*	10.86	11.11
L08246	induced myeloid leukemia cell differentiation protein	*MCL-1*	10.98	12.29
M60974	growth arrest and DNA-damage-inducible protein	*GADD45*	11.03	10.77
V00530	hypoxanthine-guanine phosphoribosyltransferase	*HPRT*	11.10	14.01
U13737	caspase-3	*CASP3*	11.35	9.94
U28014	caspase-4 precursor	*CASP4*	11.61	10.28
U78798	TNF receptor-associated factor 6	*TRAF6*	12.18	11.77
U21092	CD40 receptor-associated factor 1	*CRAF1*	12.76	10.20
U45878	inhibitor of apoptosis protein1	*HIAP1*	13.05	9.09

Apoptosis. To analyze the mechanism of cell death induced by these agents, apoptosis (programmed cell death) was analyzed and the results are presented in Figure 4. Flow cytometric assay showed that apoptosis is one of the major events leading to tumor cell death. The percentage of Annexin V-EGFP- and propidium iodide-positive cells increased with escalating doses of curcumin. The MCF-7 cell line had a significantly higher percentage of curcumin-induced apoptosis than the MCF-10A cells. The percentage of apoptotic cells increased from 0.8 to 92.6% in MCF-7 cells and from 0.5 to 53.9% in MCF-10A cells with escalating curcumin concentrations of 0-100 μg/ml.

Apoptotic gene expression by microarray hybridization. The gene expression changes induced by curcumin during apoptosis were studied using microarray hybridization of Clonetech apoptosis membrane arrays (214 genes). Curcumin concentrations around IC_{50} values (25 and 50 μg/ml) were selected for microarray hybridization. The analysis of microarray data showed that the gene expression changes induced by curcumin (25 and 50 μg/ml) were relatively insignificant in the human mammary epithelial (MCF-10A) cell line compared to changes in the breast cancer (MCF-7) cell line. There were several genes whose expression levels changed 13- to 14-fold in the MCF-7 cell line compared to only 0.65- to 1.5-fold in the MCF-10A cell line upon exposure to curcumin (Figure 5). Changes in the expression of several candidate genes of apoptosis were observed. Analysis of gene expression data using the EPCLUST program showed that, out of the 214 apoptosis-associated genes in the array, the expression of 104 genes was altered by curcumin treatment. Of these, 22 genes were up-regulated (>3-fold) at both 25 μg/ml and 50 μg/ml curcumin in the MCF-7 cell line (Table I). The up-regulated genes included CD40 receptor-associated factor 1, inhibitor of apoptosis protein 1, TNF receptor-associated factor 6, caspase-4 precursor, caspase-3, hypoxanthine-guanine phosphoribosyl transferase, growth arrest and DNA-damage inducible protein, induced myeloid leukemia cell differentiation protein, caspase-2 precursor, apoptosis regulator bclw, capase-1, tumor necrosis factor type 2 receptor-associated protein, glutathione S-transferase pi, death-associated protein 6, p53-inducible proteins 3, 6 and 11, ubiquitin, proline dehydrogenase, proliferating cell nuclear antigen, CDC10 protein homolog, c-jun N-terminal kinase 1 and RBP2 retinoblastoma-binding protein. Curcumin, at both 25 and 50 μg/ml doses, down-regulated 17 apoptotic genes in the array (<3-fold). These included tumor necrosis factor-related apoptosis-inducing ligand, caspase-7 precursor, tumor necrosis factor receptor,

Table II. *Genes down-regulated at both curcumin doses (<3-fold).*

Gene bank number	Protein/gene name	Gene symbol	Curcumin (25 μg/ml)	Curcumin (50 μg/ml)
U57059	TNF-related apoptosis inducing ligand	*TRAIL*	–14.45	–15.15
U37448	caspase-7 precursor	*CASP7*	–14.02	–2.26
M32315	tumor necrosis factor receptor	*TNFR*	–13.88	–14.58
U45880	inhibitor of apoptosis protein 3	*API3*	–13.11	–13.81
M31159	insulin-like growth factor-binding protein 3 precursor	*IGFBP3*	–13.09	–13.79
AF017988	secreted apoptosis-related protein 3	*SARP3*	–13.08	–3.41
M63167	protein kinase B	*PKB*	–12.68	–13.38
U56390	caspase-9 precursor	*CASP9*	–12.45	–3.91
D25216	insulin-like growth factor-binding protein complex acid labile chain	*IGFBP*	–11.69	–12.39
D38122	apoptosis antigen ligand	*TNFSF6*	–11.50	–4.26
AF016268	cytotoxic TRAIL receptor 2	*TRICK2A*	–11.11	–11.81
U33286	cellular apoptosis susceptibility protein	*CAS*	–10.58	–11.28
U90875	cytotoxic ligand TRAIL receptor	*TRAIL-R2*	–10.48	–3.26
U37688	retinoic acid activated cDNA	*RATS1*	–10.29	–10.99
U77845	TRAF-interacting protein	*TRIP*	–9.87	–3.70
D12614	tumor necrosis factor-beta	*TNFb*	–9.65	–10.35
Y09392	WSL protein + TRAMP + Apo-3 + death domain receptor 3	*WSL-1*	–9.50	–10.20

Table III. *Genes down-regulated by curcumin at 25 μg/ml and up-regulated at 50 μg/ml (>3-fold).*

Gene bank number	Protein/gene name	Gene symbol	Curcumin (25 μg/ml)	Curcumin (50 μg/ml)
U20536	caspase-6 precursor	*CASP6*	–10.06	4.00
U15174	Bcl2/adenovirus E1B 19kDa interacting protein 3	*NIP3*	–9.17	3.45
U90313	glutathione-S-transferase homolog	*GST*	–8.95	4.66

inhibitor of apoptosis protein 3, insulin-like growth factor-binding protein 3 precursor, secreted apoptosis-related protein 3, protein kinase B, caspase-9 precursor, IGF-binding protein complex labile chain, apoptosis antigen ligand, cytotoxic TRAIL receptor 2, cellular apoptosis susceptibility protein, cytotoxic ligand TRAIL receptor, retinoid acid activated cDNA, TRAF-interacting protein, tumor necrosis factor-beta and tumor necrosis factor receptor superfamily member 25 (Table II).

Three genes, caspase-6 precursor, Bcl2/adenovirus E1B 19kDa interacting protein 3 and glutathione-S-transferase homolog, were down-regulated (<3-fold) at 25 μg/ml curcumin and up-regulated (>3-fold) at 50 μg/ml (Table III). In addition, 40 genes showed no change at the 25 μg/ml dose while 50 μg/ml curcumin induced up-regulation (>3-fold) (data not shown). Similarly 11 genes in the array were down-regulated at the 25 μg/ml dose, with no change at 50 μg/ml curcumin (Table IV). Further, 25 μg/ml curcumin induced up-regulation of 11 genes, but the 50 μg/ml dose failed to induce any noticeable change (Table V).

Discussion

Several previous *in vitro* and *in vivo* studies have shown the preventive and therapeutic uses of curcumin against human malignancies. Recently, Aggarwal *et al.* (14) reviewed pre-clinical and clinical studies on the anticancer properties of curcumin. Moreover, chemoprevention trials with this agent have been initiated at cancer centers in the University of Arizona, Phoenix, AZ, the University of Michigan, Ann Arbor, MI and at the Cancer Institute of New Jersey, U.S.A. Furthermore, a phase II trial to evaluate the effect of curcumin on advanced pancreatic cancer patients has been started at the University of Texas M.D. Anderson Cancer Center, Houston, TX, U.S.A. Although the antitumor effects of curcumin have been investigated extensively, the overall effect of curcumin on all genes associated with tumor cell death has not been thoroughly studied in breast cancer cells. Further, the effect of curcumin on the entire cell death process, both in tumor as well as normal cells, may be useful for the ongoing preclinical and clinical investigations on this potential chemopreventive agent.

Table IV. *Genes down-regulated only at 25 μg/ml curcumin (<3-fold).*

Gene bank number	Protein/gene name	Gene symbol	Curcumin (25 μg/ml)	Curcumin (50 μg/ml)
M74816	clusterin precursor	*CLU*	–12.57	1.82
D15057	defender against cell death 1	*DAD1*	–12.53	1.85
X01394	tumor necrosis factor precursor	*TNFA*	–11.80	–0.77
U59863	TRAF-interacting protein	*I-TRAF*	–11.41	–0.35
AF010316	p53-inducible protein 12	*PIG12*	–10.46	–0.03
AF010312	p53-inducible protein 7	*PIG7*	–10.37	–1.18
U60520	caspase-8 precursor	*CASP8*	–10.10	1.06
M29645	insulin-like growth factor II	*IGF2*	–6.07	1.76
S57153	retinoblastoma-binding protein 1	*RBP1*	–5.74	–0.83
L27211	cyclin-dependent kinase 4 inhibitor	*CDK4I*	–3.78	–1.85
U23435	Abl interactor 2	*Abi-2*	–3.55	–0.54

Table V. *Genes up-regulated only at 25 μg/ml curcumin (>3-fold).*

Gene bank number	Protein/gene name	Gene symbol	Curcumin (25 μg/ml)	Curcumin (50 μg/ml)
U25265	dual specificity mitogen-activated protein kinase kinase 5	*MAPKK5*	3.32	0.09
L23959	DRTF1-polypeptide 1	*DP1*	3.55	–0.46
M83221	reticuloendotheliosis viral oncogene homolog B	*RELB*	4.46	–1.10
L41690	tumor necrosis factor receptor 1-associated death domain protein	*TRADD*	11.15	–1.10
U08015	transcription factor NF-ATc	*NF-ATc*	11.51	–1.10
U69108	TNF receptor-associated factor 5	*TRAF5*	11.72	–1.10
U91985	DNA fragmentation factor 45	*DFF45*	12.14	–1.10
U69611	TNF-alpha converting enzyme	*TACEA*	12.56	–1.10
X76104	death-associated protein kinase 1	*DAPK1*	13.01	–1.10
U18321	ionizing radiation resistance-conferring protein+death-associated protein 3	*DAP3*	13.08	–1.10
X04434	insulin-like growth factor I receptor	*IGF1R*	14.00	–1.10

In the present investigation, both turmeric and curcumin demonstrated cytotoxicity in breast carcinoma cells, although curcumin had lower IC (inhibitory concentration) values than the whole turmeric. Curcumin can induce approximately 2-fold higher levels of cell death in breast carcinoma cells than turmeric. When the breast carcinoma (MCF-7) and human mammary epithelial cell lines (MCF-10A) were compared, it was clear that the MCF-10A cells are less sensitive to turmeric and curcumin than former. Since a difference in the color of the media between MCF-7 and MCF-10A cells was observed, the concentration of curcumin retained in the media was estimated after 24 h in a spectrophotometer. The MCF-7 media contained significantly higher levels of curcumin than that of MCF-10A cells. The absorption of curcumin appears to be similar between MCF-7 and MCF-10 cells, because the intracellular curcumin values were not significantly different between them. It appears that MCF-10A cells may be able to induce degradation of curcumin resulting in less active compounds in the medium to cause cell death. The apoptosis assay also showed a dose-dependent activation of cell death with curcumin, although the difference in estimates between MCF-7 and MCF-10A cells was very high as compared with the cytotoxicity estimates. The level of apoptosis induced by curcumin was significantly higher in MCF-7 cells than in MCF-10A cells at every curcumin concentration tested in the present study. The difference between apoptosis and cytotoxicity estimates may be due to the fact that cytotoxicity estimates include all forms of dead cells such as apoptotic and necrotic cells, whereas the apoptotic assay measures only Annexin-V-positive cells. Overall, this preferential curcumin cytotoxicity of breast cancer cells compared to human mammary epithelial cells may be quite advantageous for breast cancer prevention and therapy. This may be explained by the gene expression profiles of both these cell lines that may determine their tumorogenicity and curcumin sensitivity (11, 22, 23).

Many cancer biologists believe that tumor cells use multiple signaling pathways to escape host-defense mechanisms. Therefore, a drug or compound that is specific

to only one signaling pathway in tumor cells may not be sufficiently successful with single agent therapy. The analysis of microarray data showed that curcumin inhibits cell growth, cell proliferation and promotes apoptosis in breast cancer cells through the modulation of multiple signaling mechanisms (24). In this context, no natural agent other than curcumin has been described in the literature that can modulate multiple signal transduction pathways (3, 25-31). Moreover, phase II clinical trials have shown that curcumin is not toxic to humans even up to 8000 mg/day when taken orally for three months. The main problem associated with curcumin is its rapid clearance from the body and bioavailability (32, 33). However, whether rapid clearance of curcumin from the body is disadvantageous or advantageous is currently open to question.

Microarray data analysis showed a set of apoptotic genes up- or down-regulated at lower and higher doses (Table I and II). The regulation of multiple signaling genes associated with apoptosis (*JNK1, TRAF, AKT, MAPKK5, PKB*) as well as genes at the up-stream events of apoptosis (*P53, PIG3, PIG11, GADD45, CASP1, CASP3, CASP4*) by curcumin was quite evident from microarray data. The down-regulation of genes which will work against apoptosis, such as *IGFBP3, API, GAS* and *TRIP*, was also revealed by the gene expression profile data. Three genes, caspase-6 precursor, Bcl2/adenovirus E1B interacting protein 3 and glutathione-S-transferase homolog, were down-regulated at lower doses of curcumin and up-regulated at higher doses (Table III). Additionally, there were several apoptotic genes down- or up-regulated at one particular concentration of curcumin and not the other (Table IV and V). Some genes like *CRADD, CASP10, GADD153, CRADDBAG1, BAX, FLAME-1, BCL2, PIG10* and *NF-ÎB* were up-regulated only at 50 μg/ml curcumin, not at 25 μg/ml dose (data not shown). Similarly, some genes like *MAPKK5, TRADD, NF-ATc, TRAF5, DAP3* and *IGF1R* were up-regulated at the lower but not at the higher dose of curcumin. These differences in gene expression patterns have been reported earlier with curcumin (34) and growth factors (35). Since curcumin regulates an array of cellular biochemical processes, either positively or negatively, because of its effects on transcription factors such as *AP1, SP1, NF-ÎB*, *etc.*, the observed variation in gene expression profiles of curcumin-treated breast cancer cells can be expected (14). This concentration-dependent regulation of gene expression may be quite significant for determining the correct therapeutic regimen and, therefore, microarray hybridization data may turn out to be critical for the determination of optimum dosage in clinical trials.

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