Anti-Invasive Gene Expression Profile of Curcumin in Lung Adenocarcinoma Based on a High Throughput Microarray Analysis

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ABSTRACT

Curcumin has been reported to exhibit anti-invasive and/or antimetastatic activities, but the mechanism remains unclear. In this study, microarray analysis of gene expression profiles were used to characterize the anti-invasive mechanisms of curcumin in highly invasive lung adenocarcinoma cells (CL1–5). Results showed that curcumin significantly reduces the invasive capacity of CL1–5 cells in a concentration range far below its levels of cytotoxicity (20 μM) and that this anti-invasive effect was concentration dependent ($10.17 \pm 0.76 \times 10^3$ cells at 0 μM; $5.67 \pm 1.53 \times 10^3$ cells at 1 μM; $2.67 \pm 0.58 \times 10^3$ cells at 5 μM; $1.15 \pm 1.03 \times 10^3$ cells at 10 μM; $P < 0.05$) in the Transwell cell culture chamber assay. Using microarray analysis, 81 genes were down-regulated and 71 genes were up-regulated after curcumin treatment. Below sublethal concentrations of curcumin (10 μM), several invasion-related genes were sup-pressed, including matrix metalloproteinase 14 (MMP14; 0.65-fold), neuronal cell adhesion molecule (0.54-fold), and integrins α6 (0.67-fold) and β4 (0.63-fold). In addition, several heat-shock proteins (Hsp) [Hsp27 (2.78-fold), Hsp70 (3.75-fold), and Hsp40-like protein (3.21-fold)] were induced by curcumin. Real-time quantitative reverse transcription-polymerase chain reaction, Western blotting, and immunohistochemistry confirmed these results in both RNA and protein levels. Curcumin (1 to 10 μM) reduced the MMP14 expression in both mRNA and protein levels and also inhibited the activity of MMP2, the down-stream gelatinase of MMP14, by gelatin zymographic analysis. Based on these data, it can be concluded that curcumin might be an effective antimetastatic agent with a mechanism of anti-invasion via the regulation of certain gene expressions.

Curcumin, or diferuloylmethane, is a major chemical component of turmeric (*Curcuma longa*) and is used as a spice to give a specific flavor and yellow color to curry. It is also used as a cosmetic and in some medical preparations (Govindarajan, 1980). Curcumin has been shown to display anticarcinogenic properties in animals, as indicated by its ability to inhibit phorbol ester-induced skin tumors in a mouse model system (Huang et al., 1988). In addition to its anticarcinogenic effect, curcumin is also shown to exhibit anti-inflammatory, antiproliferative, antiangiogenic, and antioxidant properties (Aggarwal et al., 2003). These effects of curcumin may be mediated by its inhibitory effect on a host of cell-signaling factors, including AP-1 transcription factor, c-Myc, Egr-1, NF-κB, protein kinase C, epidermal growth factor receptor tyrosine kinase, c-Jun N-terminal kinase, protein tyrosine kinases, protein serine/threonine kinases, and IκB kinase (Huang et al., 1988; Hong et al., 1999; Lin et al., 2000). In our previous study, it was also found that curcumin could inhibit cell cycle progression and induce cell apoptosis by regulating the gene expression of c-myc and bcl-2 (Chen and Huang, 1998).

ABBREVIATIONS: NF-κB, nuclear factor-κB; DMEM, Dulbecco's modified Eagle's medium; PI, propidium iodide; PCR, polymerase chain reaction; EST, expressed sequence tag; IMAGE, Integrated Molecular Analysis of Genomes and their Expression; RTQ-RT-PCR, real-time quantitative reverse transcription-polymerase chain reaction; TBP, TATA box binding protein; HSP, heat shock protein; NCAM, putative neuronal cell adhesion molecule; MMP, matrix metalloproteinase; SOM, self-organizing map; ANOVA, analysis of variance; TOPO, topoisomerase; AP-1, activator protein 1.

The prognosis of cancer is mainly determined by the invasiveness of tumor cells; the metastasis of cancer cells is one of the major deciding factors in cancer outcome. Although there are already several drugs available to control cancer growth, there are still no drugs presently available that specifically inhibit the metastatic behavior of cancer cells. Curcumin has previously been identified to exhibit antimetastatic activity (Menon et al., 1995, 1999). Oral administration of curcumin at a concentration of 200 nmol/kg body weight was found to significantly inhibit lung metastasis as evidenced by the reduction in the number of lung tumor nodules (80%) and an increase in life span (143.9%) (Menon et al., 1995). Curcumin treatment (10 μg/ml = 27.14 μM) significantly inhibited the invasion of B16F-10 melanoma cells across the collagen matrix of the Boyden chamber (Menon et al., 1999). A previous study also demonstrated that curcumin inhibited angiogenesis in a subcutaneous Matrigel plug model in mice (Thaloor et al., 1998). These results indicate a possible use of curcumin in arresting the metastatic growth of tumor cells, but the mechanism remains unknown.

Metastasis is a complicated multistep process that involves interactions between cancer cells and their surrounding microenvironments. Previously, a series of lung adenocarcinoma cell lines (CL1–0, CL1–1, and CL1–5) with varying degrees of invasiveness was established (Chu et al., 1997). Tracheal graft assays confirmed that cell lines with higher in vitro invasiveness had greater in vivo invasive potential. The CL1–5 cell line exhibited relatively higher invasive ability than the other lung adenocarcinoma cell lines (CL1–0 and CL1–1). Recently, these model cell lines were used to identify invasion-associated genes using cDNA microarray with colorimetric detection (Chen et al., 2001). These data demonstrated that genes related to cell adhesion, motility, angiogenesis, signal transduction, and some other expressed sequence tag genes may play significant roles in the metastatic process.

In this report, the antimetastatic activity of curcumin by the Matrigel invasion assay system was surveyed, and the curcumin-regulated gene expression in the lung adenocarcinoma cell line (CL1–5) was further characterized using cDNA microarray technique. According to these findings, curcumin may have potential as a new antimetastasis drug. By analyzing the curcumin-regulated genes in antimetastasis and comparing the invasion-associated genes that have already been identified (Chen et al., 2001), the genetic control of cancer cell metastasis could be further explored and a pathway for new anticancer drug development could be established.

Materials and Methods

Cell Lines. The human lung adenocarcinoma cell line with highly invasive capacities (CL 1–5) was grown in DMEM medium with 10% fetal bovine serum at 37°C, 20% O_2, and 5% CO_2, as described previously with some modification (Chu et al., 1997).

Cell Viability and Apoptosis. To examine the antiproliferative effects of curcumin (Sigma, St. Louis, MO), the CL1–5 cells were treated with different concentrations of curcumin (0, 1, 5, 10, and 20 μM) for 24 h. Cell viability was assessed by the trypan blue exclusion (Chen and Huang, 1998) and confirmed by the uptake of 3-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl tetrazolium bromide assays (Chen et al., 2002b).

Apoptotic cells were detected by both PI and Annexin V labeling as described previously (Chen et al., 2002a). Double-labeling was performed at 37°C as follows: PI (50 μg/ml) and Annexin V (2 μg/ml) were added to the culture medium for 2 h, then the staining was immediately examined on the Leica DMRA fluorescence microscope (Leica, Wetzlar, Germany). Annexin V staining is an established biochemical marker of apoptosis (Koopman et al., 1994; Vermes et al., 1995). The partial loss of membrane integrity or functionality is a useful criterion for distinguishing apoptotic from necrotic and living cells.

Matrigel Invasion Assay. Invasiveness of the CL1–5 cells was examined by using the membrane invasion culture system as described previously with some modification (Chen et al., 2001). Briefly, Transwell membranes (8-μm pore size, 6.5-mm diameter; Corning Costar Corporation, Cambridge, MA) were coated with Matrigel (2.5 mg/ml; BD Biosciences Discovery Labware, Bedford, MA) and dry coatings were reconstituted in DMEM for 1 to 2 h before cell passage. Cells were trypsinized, centrifuged, and resuspended at 10^7 cells/ml in DMEM containing 10% FBS. Cells were seeded onto the upper wells of precoated transwells. Lower wells of the transwells contained the same medium. After a 24-h incubation, membranes coated with Matrigel were swabbed with a Q-tip, fixed with methanol, and stained with crystal violet before cell counting under phase-contrast microscopy.

Biotinylated Probe Preparation and Microarray Hybridization. Five micrograms of mRNAs derived from CL1–5 treated with or without curcumin (1, 5, 10, and 20 μM) were labeled with biotin during reverse transcription as described in previous reports (Hong et al., 2000; Chen et al., 2001, 2002a). The microarrays (which measured 18 × 27 mm) carrying 9600 PCR-amplified cDNA fragments were prepared on nylon membranes by an arraying machine (Wittech, Taipei, Taiwan). The 9600 nonredundant EST clones were Integrated Molecular Analysis of Genomes and their Expression (IMAGE) human cDNA clones, each representing a putative gene cluster with an assigned gene name in the Unigene clustering. All hybridization experiments were performed in triplicate. The details of probe preparation, hybridization, and color development have also been described previously (Chen and Huang, 1998; Chen et al., 2001). The microarray images were scanned and digitized using a flatbed scanner (PowerLook 3000; UMAX, Taipei, Taiwan). The scanner provided 3000 dpi resolution and was suitable for larger arrays, such as arrays of 9600 elements. The microarray images were processed by commercial image processing programs to convert the true-color images into gray-scale images, and the image analysis and spot quantification were done with the use of the GenePix 3.0 program (Axon Instruments, Union City, CA).

Real-Time Quantitative RT-PCR. To confirm the expression patterns of up-regulated or down-regulated genes after curcumin treatment, the selected cell proliferation- and metastasis-related genes were chosen for further analysis using real-time quantitative reverse transcription-PCR (RTQ-RT-PCR) in a 96-well format. The detailed procedure has been described previously (Chen et al., 2003). Briefly, all reactions were carried in 50-μl volumes containing 25 μl of SYBR Green PCR master mix (Applied Biosystems Inc., Foster City, CA). The TATA box binding protein (TBP) was quantified as an internal control using the primers described in a previous report (Chen et al., 2003). DNA amplification was carried out using a ABI 7700 Sequence Detection System (Applied Biosystems), and the detection was carried out by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. After cycling, relative quantitation of tested gene cDNA against the internal control, TBP, was possible using a ΔC_T method. An amplification plot of the fluorescence signal versus cycle number was drawn. In the initial cycles of PCR, there was little change in fluorescence signal. This defined the baseline for the amplification plot. An increase in fluorescence above the baseline indicated the detection of accumulated PCR product. A fixed fluorescence threshold was set above the baseline in the exponential phase of the PCR. The parameter C_T (threshold cycle) was defined as the fractional cycle number at which the fluorescence

passed the fixed thresholds. The differences (ΔC_T) between the mean values in triplicate samples of tested gene cDNA and those of TBP were calculated by Microsoft Excel and the relative quantitation value was defined as $2 - \Delta CT \times K$, where K is a constant).

Western Blot Analysis and Immunohistochemistry. Western blotting and immunohistochemistry were used to confirm the differentially expressed genes after curcumin treatment. The detailed procedures were as described previously (Chen et al., 2002b). The primary antibodies for Hsp27, Hsp40, Hsp70, topoisomerase I, topoisomerase IIα, neural cell adhesion molecule (NCAM), AXL tyrosin kinase, and androgen receptor were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and primary antibody for MMP14 (MT1-MMP) was purchased from Chemicon (Temecula, CA). The dilutions of antibodies were 1:1000 for Western blotting and 1:100 for immunohistochemistry. For Western blot, α-tubulin was used as an internal control. Membranes were developed using the substrate of alkaline phosphatase, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate. The signal intensities for the proteins were quantified using a densitometer (Imagemaster V-DS; Amersham Biosciences, Piscataway, NJ). Fluorescein isothiocyanate-conjugated (for MMP14, Hsp40, and Hsp 70) and tetramethylrhodamine B isothiocyanate-conjugated (for NCAM and topoisomerase IIα) 2nd antibodies (Santa Cruz, Biotech Inc., CA) were used for immunohistochemistry. 4,6-Diamidino-2-phenylindole or propidium iodide was used as counter-staining for cell nuclei. The stained cells were then examined by fluorescence microscopy (DM-RA; Leica, Wetzlar, Germany).

Zymographic Analysis. Curcumin effects on MMP2, MMP9, and MMP14 gelatinolytic activity were assayed by gelatin zymography as described previously with some modification (Dell'Aica et al., 2002). Briefly, after the curcumin treatment, the media were collected (for MMP2 and -9) and the cells were harvested and re-suspended in 1% digitonin, 0.15 M NaCl, 20 mM HEPES, pH 7.0 (DIG buffer), and incubated for 2 h at 4°C with gentle motion. The sample was then centrifuged for 5 min at 1200g to collect the cell membrane protein-containing supernatant (for MMP14). Without heating, samples (medium and membrane protein-containing supernatant) underwent electrophoresis in 7.5% polyacrylamide gels containing 0.1% gelatin in the presence of SDS under nonreducing conditions. After electrophoresis, the gels were washed twice for 15 min with 2.5% Triton X-100 and incubated overnight at 37°C in 50 mM Tris-HCl, 200 mM NaCl, 10 mM CaCl$_2$, pH 7.4 (buffer 1). The gels were then stained for 30 min with 30% methanol/10% acetic acid containing 0.5% Coomassie Brilliant blue R-250 and destained in the same solution without dye; clear bands represent areas of gelatinolysis on the blue background. Digestion bands were quantified using the densitometer (Imagemaster V-DS; Amersham Biosciences).

Transcriptional Regulation Assay. NF-κB transcriptional activity was determined by reporter gene assay, as in our previous study (Chen et al., 2003). Briefly, the CL1–5 cells (5×10^4 cells/well) were seeded into six-well plates and allowed to grow for 24 h. The cells were cotransfected with NF-κB-binding domain luciferase (pGL2–4$\times$ AGGGGACTTTCC-luciferase; a gift from Dr. Shuang-En Chung, National Health Research Institutes) and pSV-β-galactosidase constructs (to normalize the transfection efficiency) (Promega, Madison, WI) by LipofectAMINE method (Jain and Magrath, 1991). The transfected cells were treated with different concentrations of curcumin for 24 h. Cell lysate was harvested to assay the luciferase activity according to the Tropix Luciferase Assay kit (Tropix). All of the experiments included nontransfection and vector alone because negative controls were performed in triplicate and normalized with both transfection efficiency and protein quantity.

Statistical Analysis. Cluster analysis was performed to identify curcumin-regulated genes on the microarray. Gene expression data obtained from the microarray experiments were processed and normalized using the protocol and program described by Iyer et al. (1999). Genes were clustered into groups on the basis of expression profiles by the self-organizing maps (SOMs) algorithm as described

by Tamayo et al. (1999) and previous studies conducted by the authors (Chen et al., 2001). After cluster analysis by the SOM method, genes whose expression profiles correlated either positively or negatively with the concentrations of curcumin were identified. Genes whose expression correlated with the invasiveness of cell lines were also grouped into categories by their putative functions on the basis of literature reports (Table 1).

A repeated-measures ANOVA was performed to determine any significant differences between the invasive cells and mRNA or protein expression intensities. Data from at least three independent experiments were analyzed by ANOVA (Excel, Microsoft, Taipei, Taiwan, Republic of China) to determine any significant difference.

Results

Low Concentrations of Curcumin Can Suppress Cell Invasion. The number of viable cells after curcumin treatment was estimated using a hemocytometer with the trypan blue dye exclusion test. Exposure of CL1–5 cells to curcumin (0, 1, 5, 10, 20, 50, and 60 μM) influenced the cell viability of CL1–5 cells in a concentration-dependent manner (92.3 $\pm$ 6.1% at 10 μM, 70.1 $\pm$ 7.3% at 20 μM, and 29.6 $\pm$ 2.8% at 30 μM, $P < 0.05$, ANOVA) (Fig. 1A). The IC$_{50}$ of curcumin was 33.2 $\pm$ 3.7 μM in CL1–5 cell viability ($n = 4$). Furthermore, curcumin concentration-dependently ($\geq$20 μM in CL1–5 cells) induced cell apoptosis, as examined by flow cytometry and fluorescence microscopy with Annexin-V/PI (Fig. 1B). These results indicate that curcumin exhibited antiproliferative activity and induced cell apoptosis in CL1–5 cells.

Metastasis is highly dependent on tumor cell invasion. Therefore, the effect of curcumin on CL1–5 was determined. For the invasion assay, CL1–5 cells were penetrated through a uniform layer of Matrigel, which consists of collagen type IV, heparan sulfate proteoglycan, entactin, and laminin. The results showed that curcumin (1 to 10 μM) could significantly inhibit cell invasion of CL1–5 cells (56.0 $\pm$ 16.1% at 1.0 μM, $P < 0.05$) in a concentration-dependent manner with an IC$_{50}$ value of 1.2 $\pm$ 0.3 μM (Fig. 1A). It also demonstrated that curcumin could inhibit cell invasion at a concentration lower than the dose that induced cell apoptosis (20 μM) (Fig. 1). According to these results, concentrations of 0, 1, 5, 10, and 20 μM were used for microarray analysis and the following experiments.

Microarray Analysis of Gene Expression of Lung Cancer Cells after Treatment with Curcumin. Biotin-labeled probe derived from CL1–5 cell line mRNA treated with or without curcumin were hybridized to microarrays, with 9600 putative genes to profile the curcumin-related gene expression patterns. To identify the curcumin-related anti-invasive genes, cluster analysis was performed on the expression profiles of the curcumin-treated CL1–5 cells at concentrations of 0, 1, 5, 10, and 20 μM. Of the 9600 putative genes, only 416 genes had statistically significant expression differences between normal and curcumin-treated cells.

After SOM clustering, these genes were grouped into nine clusters. Two clusters of expression profiles with descending trend containing 81 genes and two for ascending trend containing 71 genes were selected from these nine clusters. To avoid confusion of negative values in expression patterns, the scale value of normalization, from -1 to $+1$, was shifted to a positive value by adding 1 for each data, which made the scale value become 0 to $+2$. Figure 2A, left, contains two clusters of gene expression profiles that were inhibited by

TABLE 1
Curcumin-regulated genes

Acc. No.	Gene Name	Category
Curcumin-Inhibited Genes		
AI087216	Cyclin-dependent kinase 4	Cell cycle regulators
R54354	CD81 Antigen	Cell-surface antigens & cell adhesion proteins
XM_030120	Butyrophilin protein (LOC90227)	Cell-surface antigens & cell adhesion proteins
H26162	Integrin, $\alpha6$	Cell-surface antigens & cell adhesion proteins
NM_000213	Integrin, $\beta4$	Cell-surface antigens & cell adhesion proteins
NM_004995	Matrix metalloproteinase 14 (MT1-MMP)	Cell-surface antigens & cell adhesion proteins
AF063936	Putative neuronal cell adhesion molecule (NCAM)	Cell-surface antigens & cell adhesion proteins
NM_003286	DNA topoisomerase I	DNA synthesis, repair & recombination proteins
NM_001067	DNA topoisomerase II α	DNA synthesis, repair & recombination proteins
AK057293	Chromogranin A	Growth factors, cytokines & chemokines
R60197	Latent TGF-β	Growth factors, cytokines & chemokines
W45557	IGF-binding protein 4	Growth factors, cytokines & chemokines
AA206052	Myeloid leukemia factor 2	Growth factors, cytokines & chemokines
XM_012444	Neogenin (chicken) homolog 1	Growth factors, cytokines & chemokines
AI524696	Vascular endothelial growth factor B	Growth factors, cytokines & chemokines
NM_000044	Androgen receptor	Hormone receptors
NM_013360	Coagulation factor III (thromboplastin, tissue factor)	Intracellular signal transduction modulators & effectors
AI718588	Complement component (3b/4b) receptor 1	Intracellular signal transduction modulators & effectors
AI587350	G protein-coupled receptor 9	Intracellular signal transduction modulators & effectors
H16711	G protein-coupled receptor kinase 6	Intracellular signal transduction modulators & effectors
H26359	Gq protein, $\alpha11$	Intracellular signal transduction modulators & effectors
R42435	FAST kinase	Intracellular signal transduction modulators & effectors
NM 001699	AXL receptor tyrosine kinase	Intracellular signal transduction modulators & effectors
BC0001777	Hippocalcin	Intracellular signal transduction modulators & effectors
XM_035177	Protein kinase C-binding protein RACK7	Intracellular signal transduction modulators & effectors
BC015229	Cyclophilin-like protein	Intracellular signal transduction modulators & effectors
AC003024	FK-506 binding protein homologue (FKBP38)	Intracellular signal transduction modulators & effectors
AI424152	Nuclear factor of κB	Intracellular signal transduction modulators & effectors
H08653	MAP kinase 3; p44	Intracellular signal transduction modulators & effectors
AA176401	Protein phosphatase 1, regulatory (inhibitor) 3	Intracellular signal transduction modulators & effectors
AI378141	Testis-specific kinase 1	Intracellular signal transduction modulators & effectors
H14808	ATPase, Na^+/K^+ transporting, $\beta2$	Ion channel & transport proteins
AI469102	Chloride intracellular channel 1	Ion channel & transport proteins
NM_021904	GABA B receptor, 1	Ion channel & transport proteins
AI086951	Glucose-6-phosphatase, transport protein 1	Ion channel & transport proteins
BC008837	Aldo-keto reductase family 1	Metabolic pathway proteins
H05285	Glutaminyl transsynthase	Metabolic pathway proteins
AI087093	Retinol dehydrogenase	Metabolic pathway proteins
AI304696	Oxoglutarate dehydrogenase (lipoamide)	Metabolic pathway proteins
NM_004454	Prostaglandin I2 (prostacyclin) synthase	Metabolic pathway proteins
R38694	PIM-1 proto-oncogene	Oncogenes & tumor suppressors
H51148	RAB3A, member RAS oncogene family	Oncogenes & tumor suppressors
NM_004449	v-ets oncogene	Oncogenes & tumor suppressors
R51318	Translation initiation factor 3γ	Transcription factors & general DNA binding proteins
H09658	RNA polymerase II transcription factor SIII p18	Transcription factors & general DNA binding proteins
AA176383	Polymerase (RNA) II C	Transcription factors & general DNA binding proteins
H06125	Zinc finger protein 177	Transcription factors & general DNA binding proteins
R55356	Zinc-finger helicase (Snf2-like)	Transcription factors & general DNA binding proteins
XM_035177	FLJ10631	EST
AK057293	FLJ32731	EST
BC015229	IMAGE: 3898846	EST
AA551245	KIAA0100 gene product	EST
AI243749	KIAA0550 gene product	EST
R44184	KIAA0751 gene product	EST
Curcumin-Induced Genes		
NM_144781	Programmed cell death 2 (PDCD2)	Apoptosis-related proteins
T81764	Cell division cycle 27	Cell cycle regulators
AI015629	Cyclin-dependent kinase 6	Cell cycle regulators
H12306	p21/Cdc42/Rac1-activated kinase 1	Cell cycle regulators
R34064	Catenin (cadherin-associated protein), $\alpha1$	Cell-surface antigens & cell adhesion proteins
AI473129	CD39-like 4	Cell-surface antigens & cell adhesion proteins
AI469220	Protease inhibitor 2 (anti-elastase)	Cell-surface antigens & cell adhesion proteins
U07343	DNA mismatch repair protein MLH1	DNA synthesis, repair & recombination proteins
AI521477	p60 katanin	Extracellular matrix proteins/cytoskeleton & motility proteins
AI539158	TACC2 protein (TACC2)	Extracellular matrix proteins/cytoskeleton & motility proteins
R59731	Microtubule-associated protein TAU	Extracellular matrix proteins/cytoskeleton & motility proteins
W60313	Phosphatidylinositol glycan, class F	Extracellular matrix proteins/cytoskeleton & motility proteins
W96163	Transducer of erbB-2	Growth factor & chemokine receptors
AA573793	Trefoil factor 1 (estrogen-inducible)	Growth factor & chemokine receptors
H40342	Lymphocyte-specific protein 1	Inflammatory response
AA039932	Thromboxane A2 receptor	Inflammatory response
AA532703	Interleukin 10 receptor β	Interleukins & interferons

curcumin. Each of the two clusters contained expression profiles of 44 and 37 genes. Figure 2B, left, shows the expression profiles of the two clusters that were induced by curcumin. Each of these contained 24 and 47 genes.

All of the gene expression profiles in these clusters were rearranged by hierarchical cluster analysis with the average linkage method (Iyer et al., 1999; Chen et al., 2001), and the expression levels were gray-scale encoded as shown at the right of each SOM cluster (Fig. 2, A and B). Figure 2A shows the levels of gene expression from high (white) to low (black), indicating that these genes were down-regulated by curcumin; Fig. 2B shows the gene expression levels from low (black) to high (white), indicating these genes were up-regulated by curcumin.

Categories of Curcumin-Regulated Genes. These genes clustered in Fig. 2 were grouped into several categories on the basis of their cellular functions (Table 1). In Table 1, these categories included cell cycle regulators (such as cyclin-dependent kinase 4), cell-surface antigens and cell adhesion proteins (such as integrins $\alpha6$ and $\beta4$, MMP14, and NCAM), DNA synthesis, repair, and recombination proteins (such as DNA topoisomerase I and IIα), growth factors, cytokines, and chemokines (such as vascular endothelial growth factor-B), hormone receptors (such as androgen receptor), intracellular signal transduction modulator and effectors (such as G-protein coupled receptor kinase 6, AXL receptor tyrosine kinase, and FAST kinase), ion channel and transport proteins (such as ATPase, Na$^+$/K$^+$ transporting protein $\beta2$), metabolic pathway proteins (such as glutaminyl-trans synthase), oncogenes and tumor suppressors (such as v-ets), transcription factors and general DNA binding proteins (such as zinc finger pro-

tein 177), apoptosis-related proteins (such as programmed cell death 2), inflammatory response proteins (such as thromboxane A2), interleukins and interferons (interleukin 6 and 10 receptor β), extracellular matrix proteins (such as microtubule-associated protein, TAU), stress response proteins (such as Hsp40-like protein and Hsp70), and EST genes (such as FLJ10631, FLJ 32731, and IMAGE3898846).

Verification of Curcumin Regulated Genes Identified by cDNA Microarray by RTQ-RT-PCR, Western Blot, and Immunohistochemistry. To substantiate the results of the microarray studies, RTQ-RT-PCR was performed to assess the mRNA expression of 16 of the curcumin-regulated genes (10 for down-regulation and six for up-regulation). Western blotting and immunohistochemistry were used to assess the protein expression levels and localization of several of the gene products based on the availability of antibodies.

As shown in Fig. 3, A and B, the chosen genes, which were confirmed with RTQ-RT-PCR, were compared to obtain the results of different expression ratios in microarray analysis. These results showed that the mRNA levels of DDIT3 (3.82 ± 0.38-fold; increased), GRM7 (5.91 ± 0.49-fold; increased), Hsp27 (3.25 ± 0.41-fold; increased), Hsp40-like protein (2.82 ± 0.37-fold; increased), and Hsp70 (3.32 ± 0.43-fold; increased) were confirmed to be up-regulated; and GABBR1 (2.54 ± 0.02-fold; decreased), FLJ32731 (2.15 ± 0.10-fold; decreased), MMP14 (MT1-MMP) (2.01 ± 0.07-fold; decreased), NCAM (1.92 ± 0.06-fold; decreased), IMAGE clone 3898846 (1.91 ± 0.05-fold; decreased), FLJ10831 (1.87 ± 0.07-fold; decreased), TOPO I (1.34 ± 0.12-fold; decreased), TOPO IIα (1.65 ± 0.06-fold; decreased), v-ets oncogene

TABLE 1 Continued

Acc. No.	Gene Name	Category
AI074123	Interleukin 6	Interleukins & interferons
AI304505	Cryptochrome 2 (photolyase-like)	Intracellular signal transduction modulators & effectors
R43329	Protein phosphatases PP1 regulator	Intracellular signal transduction modulators & effectors
AI654758	fer (fps/fes related) tyrosine kinase	Intracellular signal transduction modulators & effectors
H06479	MacMarcks	Intracellular signal transduction modulators & effectors
R43220	Cdc14B2 phosphatase	Intracellular signal transduction modulators & effectors
R20770	Syntaxin 4 binding protein UNC-18c	Intracellular signal transduction modulators & effectors
R37588	RalGDS-like 2 (RGL2)	Intracellular signal transduction modulators & effectors
AA114872	Iron-responsive element binding protein 1	Intracellular signal transduction modulators & effectors
H11973	Potassium channel protein KV2.1	Ion channel & transport proteins
AA961937	Cytochrome c oxidase subunit VIIb	Metabolic pathway proteins
R41989	C-1-Tetrahydrofolate synthase C	Metabolic pathway proteins
AI263472	Serine palmitoyltransferase, subunit I	Metabolic pathway proteins
R37570	Ubiquitin conjugating enzyme (UbcH8)	Metabolic pathway proteins
H15721	Phosphoribosyl pyrophosphate synthetase 2	Metabolic pathway proteins
U92458	Glutamate receptor, metabotropic 7 (GRM7)	Neurotransmitter receptors
AA121523	Atrial natriuretic factor precursor	Stress response proteins
AA514500	ATX1 (antioxidant protein 1)	Stress response proteins
R82347	Glutathione S-transferase π-1	Stress response proteins
XM_058528	DNA-damage-inducible transcript 3 (DDIT3)	Stress response proteins
NM_005345	Heat shock 70-kDa protein1A (HSP70–1A)	Stress response proteins
AB020027	Heat shock 27-kDa protein (HSP27)	Stress response proteins
H81637	Putative DDB p127-associated protein	Stress response proteins
BC001144	ER-associated DNAJ (HSP40)	Stress response proteins
NM_003932	p48, Hsp70 interacting protein	Stress response proteins
R59900	E2F transcription factor 4, p107/p130-binding	Transcription factors & general DNA binding proteins
AA080948	Eukaryotic translation initiation factor 4E	Transcription factors & general DNA binding proteins
XM_028204	NFKB1	Transcription factors & general DNA binding proteins
N20235	Pre-B cell leukemia transcription factor-2	Transcription factors & general DNA binding proteins
AI138789	EST	EST
AI186064	EST	EST
AI125328	EST	EST
W90164	IMAGE:418150	EST
AI284062	KIAA0020 gene product	EST
AA280281	KIAA0171 gene product	EST

(1.39 ± 0.13-fold; decreased), and LOC90227 (1.33 ± 0.12-fold; decreased) were down-regulated at 24 h after curcumin treatment (10 μM) by RTQ-RT-PCR in CL1–5 cells.

Similarly, Western blotting confirmed that stress-response proteins (Hsp27, Hsp40-like protein, and Hsp70), were increased after curcumin treatment (Fig. 4). The cell invasion-related factors (MMP14 and NCAM) and proliferative factors (TOPO-I and IIα) were all down-regulated in both mRNA and protein levels after curcumin treatment in a concentration-dependent manner (0, 1, 5, 10, and 20 μM) (Fig. 3 and 4). As indicated by the immunohistochemical staining in Fig. 5, the

protein expression levels of NCAM, MMP14 (MT1-MMP), and TOPO-IIα were highly expressed in CL1–5 cells without curcumin treatment. The NCAM and MMP14 (MT1-MMP) proteins were localized on the cell membrane, whereas TOPO-IIα was located in the cell nuclei with granular distribution. All of these proteins were reduced by curcumin in a concentration-dependent manner (1, 5, and 10 μM curcumin). In particular, the protein expression of TOPO-IIα was not only down-regulated but also lost the granulated localization in cell nuclei after curcumin treatment in CL1–5 cells (Fig. 5). On the other hand, the expression of HSPs
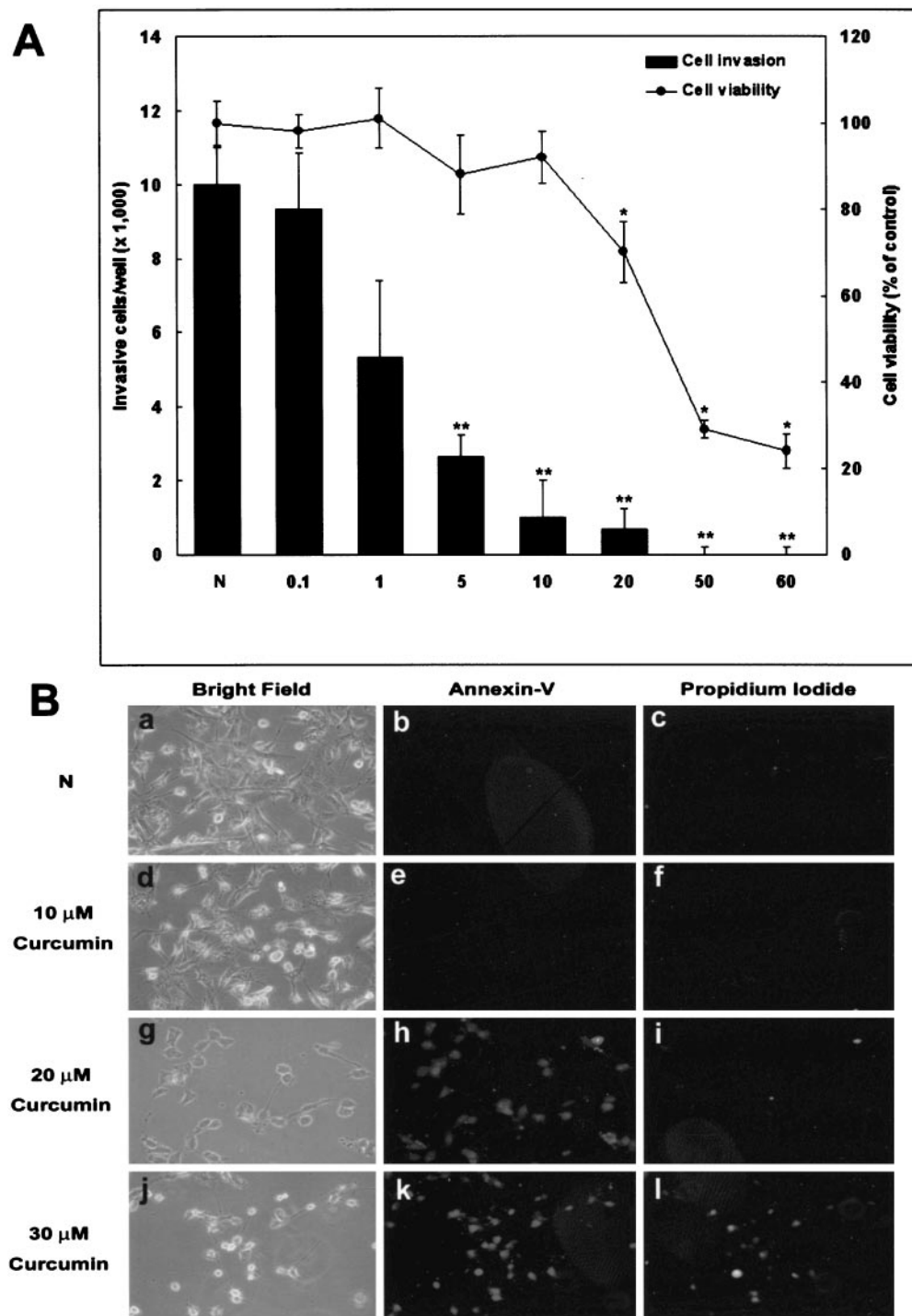

Fig. 1. Effects of curcumin on CL1–5 cell viability, cell invasion, and apoptosis after 24 h of incubation. A, the effect of curcumin (0, 1, 5, 10, 20, 50, and 60 μM) on the cell viability of CL1–5 cells was expressed as a percentage of the normal control values (% of control). Each point and vertical line represents the mean ± S.E.M. ($n = 5$). *, $P < 0.05$ compared with normal control values in the absence of curcumin (N). The bars present the in vitro invasion activity of CL1–5 cells incubated with or without different concentrations of curcumin (0, 1, 5, 10, 20, 50, and 60 μM) for 24 h. Bars, average number of cells which invaded through the matrix gel. Each column represents the mean ± S.E.M. ($n = 5$). **, $P < 0.05$, compared with normal control values in the absence of curcumin (N). B, curcumin-induced morphological change and apoptosis in CL1–5 cells. CL1–5 cells untreated (a, b, and c), or treated with 10 μM curcumin (d, e, and f), 20 μM curcumin (g, h, and i), or 30 μM curcumin (j, k, and l) were performed using PI and Annexin V labeling as described under *Materials and Methods*. Shown are bright-field views (BF; a, d, g, and j) and fluorescence microscopic observation with annexin-V (Annexin-V; b, e, h, and k) or propidium iodide (PI; c, f, i, and l) staining. Similar results were obtained in four additional experiments using independent CL1–5 cell preparations, original × 400.

(Hsp27, Hsp40, and Hsp70) were increased and the proteins were translocated from the cytosol to nuclei after curcumin treatment (Fig. 5).

Effect of Curcumin on MMP Activity. As previously reported, it has been shown that MMP14 might be associated with the tissue inhibitor of metalloproteinase 2, both acting together as a receptor for pro-MMP2 and leading to the cleavage of the zymogen in several tumor systems (Fillmore et al., 2001). The expression of MMP14 was significantly down-regulated after curcumin treatment (1 to 10 μM; Fig. 6). Furthermore, the effects of curcumin on MMP2 gelatinolytic activity were studied using the zymographic analysis. The conditioned medium of CL1–5 cells produced clear bands of degradation in the gelatin zymography (Fig. 6, Control). Significantly, curcumin showed the inhibitory effect of MMP2 (both in pro-MMP2 and MMP2, gelatinase A) activity by reducing its gelatinolytic activity, whereas the MMP9 (gelatinase B) activity (Fig. 6) and MMP14 (data not shown) were not significantly influenced by curcumin.

NF-κB Transcriptional Activity Was Inhibited by Curcumin. The NF-κB transcriptional activity was assessed by luciferase reporter gene assay. The luciferase activity in CL1–5 cells after curcumin treatment was significantly suppressed ($86.5 \pm 21.5\%$ at 5 μM curcumin, $P > 0.05$; $42.4 \pm 8.2\%$ at 10 μM curcumin, $P < 0.05$, compared with control). The mock-transfected and nontransfected control cells reveled no response in luciferase activity assay (Fig. 7).

Discussion

The anticancer mechanism of curcumin is diverse. Curcumin has been reported to exhibit antiproliferative, antiangiogenic, anti-inflammatory, and antioxidative properties through down-regulating transcription factors NF-κB, AP-1, c-Myc, and Egr-1; to reduce expression of vascular endothelial growth factor, angiopoietin 1 and 2; and to inhibit the activity of c-Jun N-terminal kinase (Chen and Huang, 1998; Hong et al., 1999; Gururaj et al., 2002; Aggarwal et al., 2003). In this study, we found that low concentrations of curcumin could reduce the cancer cell invasive activity. The gene expression profile of curcumin-induced anti-invasive effect was analyzed genome-wide by cDNA microarray. This panel of genes that are regulated by low concentration of curcumin might partially explain the curcumin-induced anti-invasive mechanism. This gene expression pattern may further bring insights into the action mechanisms of curcumin and provide the information to design more effective antitumor treatments.

Degradation of extracellular matrix by MMPs is crucial for malignant tumor growth, invasion, metastasis, and angiogenesis. Thus, the MMPs have been suggested as potential prognostic markers and therapeutic targets in cancer (Nagase and Woessner, 1999). MMP14 (MT1-MMP), a membrane-type MMP, is anchored to the cell membrane to degrade ECM components (collagen I, II, III, gelatin, laminin, etc.). The high expression level of MMP14 (MT1-MMP) is related to tumor invasion capacity (Vihinen and Kahari,
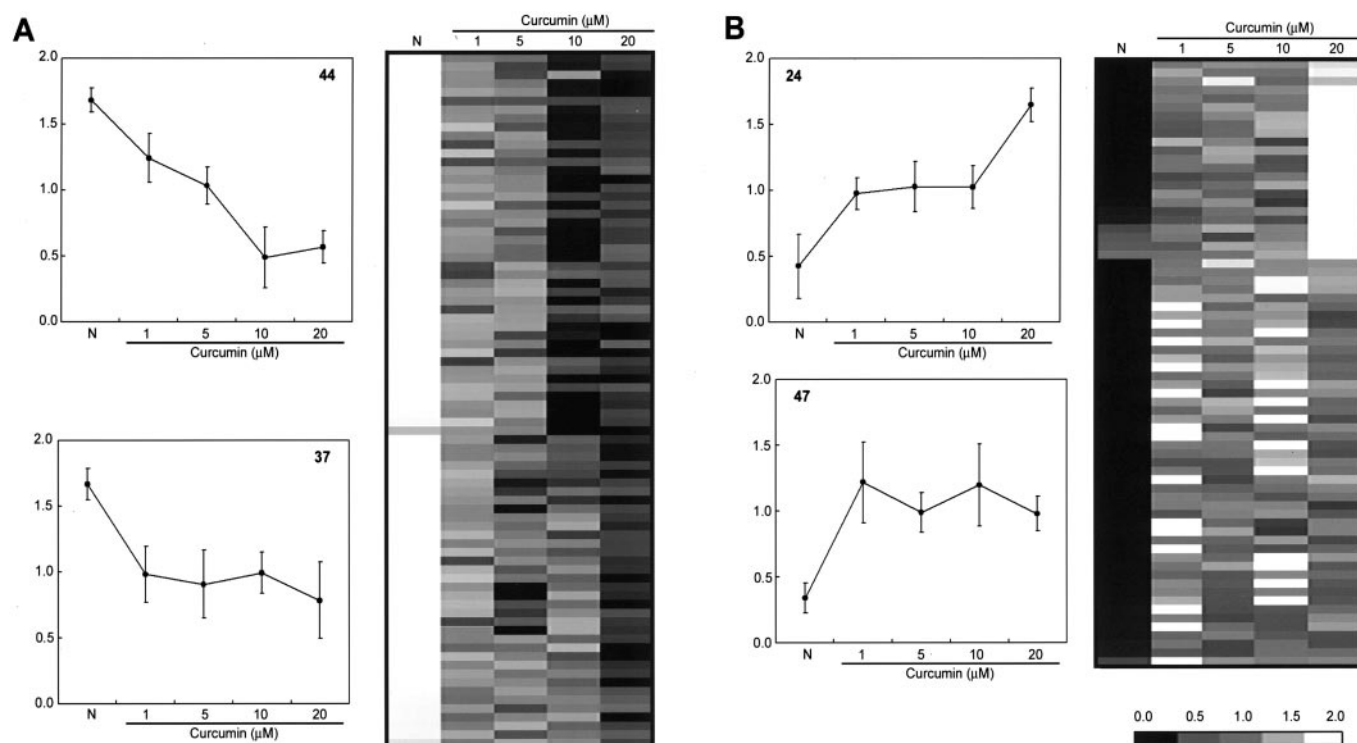
Fig. 2. Cluster analysis of expression profiles of CL1–5 cells treated with different concentrations of curcumin (0, 1, 5, 10, and 20 μM). A, the genes with descending trend after curcumin treatment. Left, SOM clustering. Two clusters were selected from nine clusters. The number of genes in each cluster is indicated at the corner of each panel, and each cluster was represented by the centroid. Right, hierarchical clustering. All the genes derived from the two descending trend SOM clusters (comprising 44 and 37 genes) were clustered by the average linkage method, and the cluster image shows the trend of gene expression levels with (1, 5, 10, and 20 μM) or without (N) curcumin treatment. (B) The genes with ascending trend after curcumin treatment. B, left, SOM clustering. Right, hierarchical clustering.

2002). In the present study, CL1–5 cells exhibit high levels of expression MMP14 (MT1-MMP) and were down-regulated after curcumin treatment.

MMP-2 (gelatinase A) is believed to be of crucial importance in processes requiring basement membrane disruption, such as tumor invasion and metastasis (Bergman et al.,

2003). Overexpression of MMP14 mRNA was reported in tumoral tissues and correlated to the activation of MMP2 in brain, head and neck, breast, and gastrointestinal cancers (Théret et al., 1998). In the present study, the gelatinolytic activity of MMP2 could be inhibited by curcumin. This might be associated as well with the down-regulation of MMP14
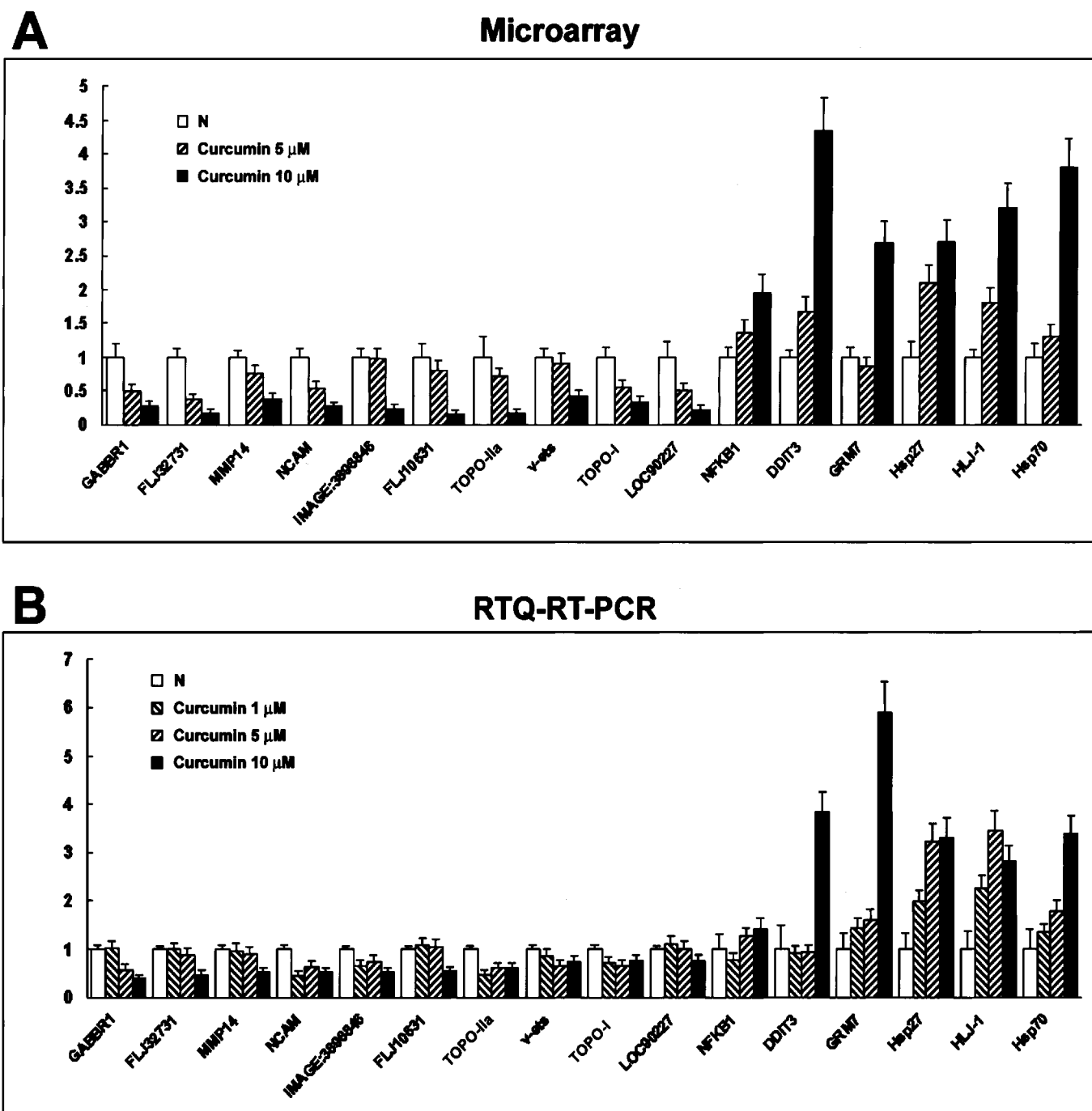
Fig. 3. Comparison of the data of microarray analysis with real time quantitative RT-PCR. A, microarray analysis. The -fold changes of curcumin-regulated genes compared with untreated controls were shown by cDNA microarray analysis. Each column represents the mean of-fold ± S.E.M. ($n = 3$). *, $P < 0.05$, compared with normal control values in the absence of curcumin (N). B, RTQ-RT-PCR confirmed commonly up-regulated (six genes) or down-regulated (10 genes) by curcumin in CL1–5 cells. The -fold change was displayed as relative to normalized normal CL1–5 cells without curcumin treatment ($n = 3$). *, $P < 0.05$. GABBR1, γ-aminobutyric acid B receptor 1; FLJ32731 (EST); MMP14 (MT1-MMP) matrix metalloproteinase 14; NCAM, putative neuronal cell adhesion molecule; IMAGE 3898846 (EST); FLJ10631 (EST); TOPO IIα, topoisomerase IIα; TOPO I, topoisomerase I; v-ets (oncogene); LOC90227 (EST); NFKB1, nuclear factor of κ light polypeptide gene enhancer in B-cells 1 (p105); DDIT3, DNA-damage-inducible transcript 3; GRM7, glutamate receptor metabotropic 7; Hsp27, 27-kDa heat shock protein; HLJ-1, 40-kDa heat shock protein-like protein; Hsp70, 70-kDa heat shock protein.

expression by curcumin. Most of the MMP genes inducible by extracellular stimuli (MMP1, -3, -7, -9, -10, and -13) harbor an AP-1 binding site in the proximal promoter that could be inhibited by curcumin (Onodera et al., 2002). Therefore, the results of microarray analysis are reasonable in that that curcumin could inhibit MMP7 and MMP10 gene expression. However, MMP14 gene does not contain AP-1 elements (Vi-

hinen and Kahari, 2002). The mechanism whereby curcumin could regulate MMP14 (MT1-MMP) expression might be an AP-1 independent pathway, but this requires further substantiation.

Cell adhesion molecules have been demonstrated to play important roles in tumor metastasis (Skubitz, 2002). Subsequent to cDNA microarray screening, several adhesion mol-
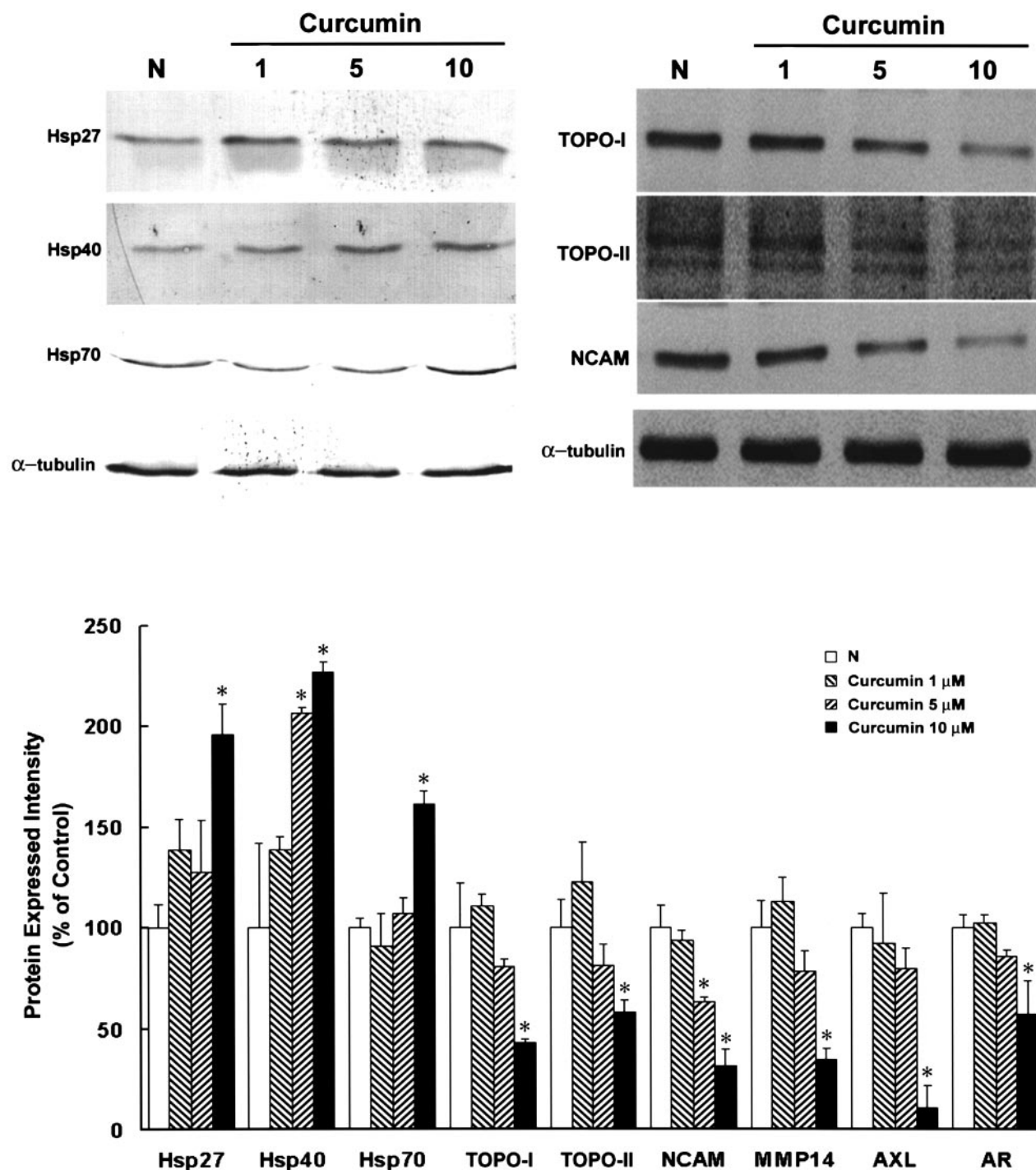

Fig. 4. Western blotting analysis. A, Western blotting confirmed commonly up-regulated or down-regulated by curcumin in CL1–5 cells. The protein levels of Hsp27, -40, and -70 were increased significantly, whereas the α-tubulin, the internal control, showed no difference in the expression level. The TOPO-I, TOPO-IIα, NCAM, MMP14 (MT1-MMP), AXL tyrosine kinase, and androgen receptor (AR) were all concentration-dependently down-regulated by curcumin (1, 5, and 10 μM). Similar results of the blotting images were obtained in four additional experiments using independent CL1–5 cell preparations. B, the data on the bar chart were expressed as a percentage of the normal control values (% of control). Each point with a vertical line represents the mean ± S.E.M. ($n = 5$). *, $P < 0.05$, compared with normal control values in the absence of curcumin (N).

ecules that were down-regulated by curcumin have been identified, including integrin α6 and β4, and a putative NCAM. In these genes, NCAM was confirmed to be reduced in both mRNA and protein levels after curcumin treatment. NCAM had been originally detected in the normal and tumoral pituitary (Jin et al., 1991). This membrane glycoprotein of the immunoglobin superfamily promotes cell-cell adhesion via a homophilic binding mechanism and is related to tumor growth rate and malignancy (Rutishauser and Landmesser, 1996). It is likely that NCAM is involved in tumor cell invasion and the metastatic process by facilitating cell detachment from the primary tumor, migration, and survival in a hostile environment (Franca et al., 2001).

DNA topoisomerases are enzymes that catalyze the passage of individual DNA strand (type I) or double helices (type II) through one another, manifested in the interconversions between topological isomers of DNA (Roth et al., 1998). These enzymes have important roles in the replication, recombination, transcription, chromosome condensation, and the maintenance of genome stability. Hence, they are good targets for antineoplastic drugs (Lin et al., 2000). The inhibition of topoisomerases could induce cell apoptosis and inhibit cell cycle progression and tumorigenesis (Bronner et al., 2002). The two major DNA isomerases in human cells (TOPO-I and II) have been firmly established to be effective molecular targets for many antitumor drugs (camptothecins and actinomycin D for TOPO-I; doxorubicin, daunorubicin, mitoxantrone, and etoposide for TOPO-II) (Lin et al., 2000). Previously, curcumin has been identified to inhibit the activity of TOPO-II

and exhibit the topoisomerase-poisoning effect (Roth et al., 1998). In the present study, both mRNA and protein expression levels of TOPO-I and II were confirmed to be reduced after curcumin treatment. The transcriptional regulation of
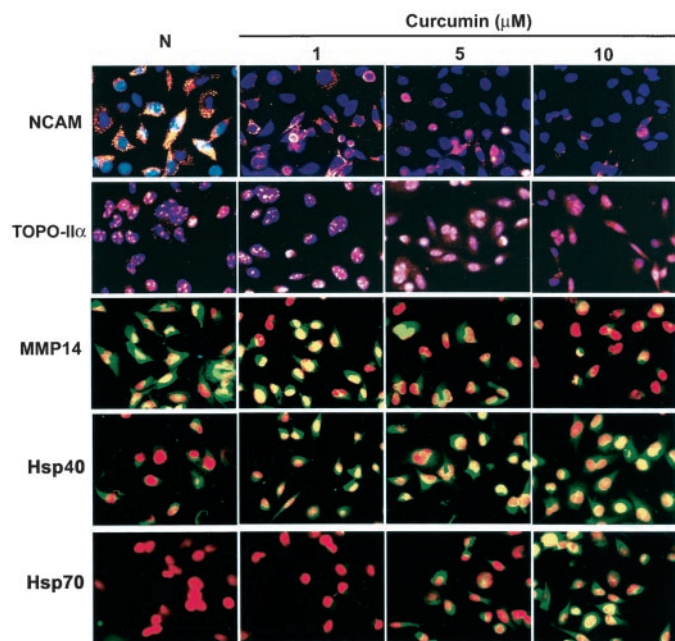

Fig. 6. Western blotting of MMP14 and zymographic analysis of MMP2 and nine activities. The CL1–5 cells were treated with (1, 5, and 10 μM) or without (N) curcumin for 24 h and the conditioned medium and cells were harvested separately for zymographic analysis. The effects of curcumin (0, 1, 5, and 10 μM) on MMP14 protein expression and also on MMP2 and MMP9 gelatinolytic activity were studied. Gelatin zymography developed in the presence of increasing concentrations of curcumin shows a progressively faint digestion band. Similar results of the zymographic images were obtained in three additional experiments.

Fig. 5. Immunohistochemical analysis. The CL1–5 cells were treated with (1, 5, and 10 μM) or without (N) curcumin, and then fixed with acetone for 5 min before staining as described under *Materials and Methods*. Specific antibodies for NCAM, MMP14 (MT1-MMP), TOPO-IIα, Hsp27, Hsp40, and Hsp70 were used in the immunostaining. After Rhodamine (orange fluorescence for NCAM or TOPO-IIα) or fluorescein isothiocyanate- (green fluorescence for MMP14, Hsp27, Hsp40, and Hsp70) conjugated secondary antibodies incubation, the cells were examined under fluorescence microscopy (Leica). 4,6-Diamidino-2-phenylindole (DAPI) (blue fluorescence) or propidium iodide (red fluorescence) was used as the counterstain of the cell nuclei. Similar results of the blotting images were obtained in four additional experiments ($n = 4$).
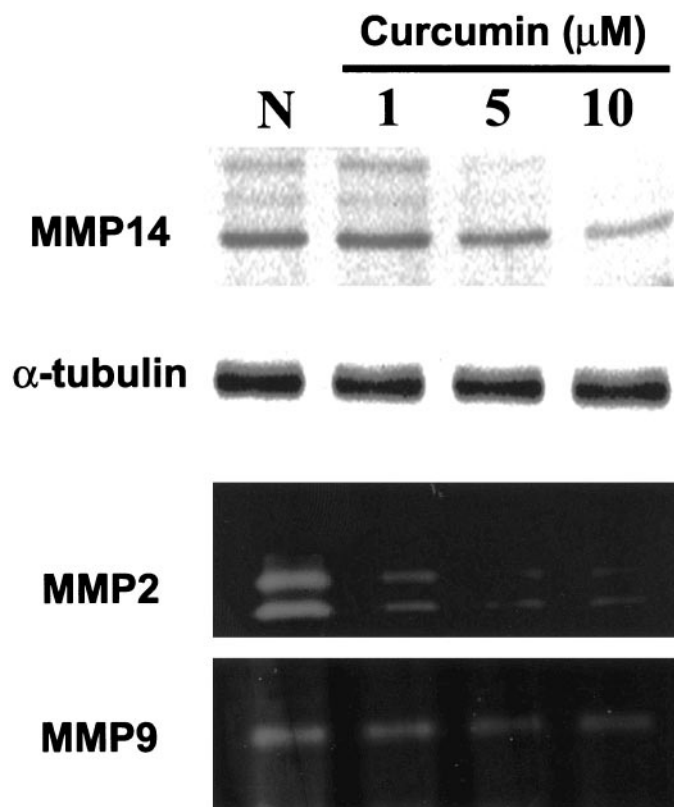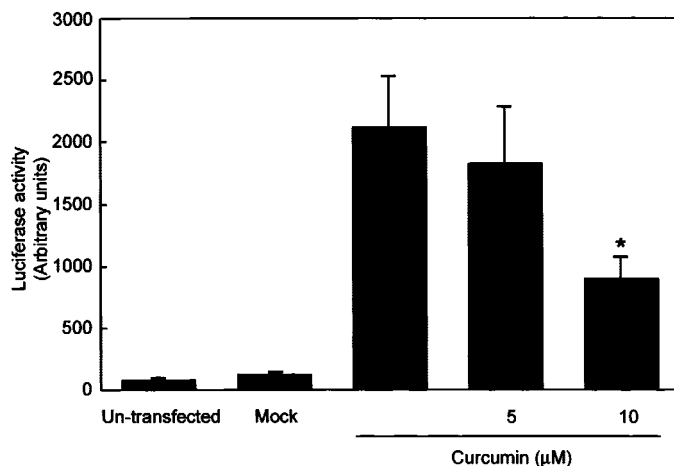

Fig. 7. Reporter gene assay of NF-κB transcriptional activity. CL1–5 cells were transfected with NF-κB binding domain luciferase construct and then treated with curcumin (5 and 10 μM). Luciferase activity assay was performed, and the results showed that it was significantly decreased in 10 μM curcumin-treated cells. *, $P < 0.05$, compared with untreated control.

TOPO I and II genes and species-specific differences are still unclear (Bronner et al., 2002).

Curcumin is a well known regulator of some certain transcriptional factors, which has been reported to inhibit AP-1 and NF-κB in transcriptional regulation in many cell systems (Philip and Kundu, 2003). The NF-κB transcriptional activity was also significantly inhibited by curcumin in CL1–5 cells. The promoters of the genes MMP-2, MT1-MMP, NCAM, and Top-IIα, showing significant down-regulation after curcumin treatment in CL1–5 cells, have AP-1 and/or NF-κB regulatory site (CTGACCTCC and/or TGGAGCTTCC) (Aggarwal et al., 2003; Bergman et al., 2003). Some of the other genes (Top-I and MT1-MMP) could be regulated by SP-1, NF-Y, YB-1, or other transcriptional factors. Previous studies also showed that SP-1 and YB-1 could be regulated by AP-1 and NF-κB. Furthermore, we also found that the expression of v-ets, the AP-1 cooperator, could be inhibited by curcumin. Our results supported the idea that curcumin could down-regulate these proteins (MT1-MMP, NCAM, Top-I, and Top-IIα) partially through inhibiting AP-1 and NF-κB. Whether other transcriptional factors or signal transductional pathways might involve in curcumin-inhibited cell invasion, further studies are needed to clarify this notion.

During carcinogenesis, HSPs have been reported to show alteration of their expression levels, either increasing or decreasing. Several findings have demonstrated that HSPs are associated with cell proliferation, differentiation, viability, apoptosis, motility, and antitumor immunity (Rousseau et al., 1997), but their clinical significance is still controversial. A statistically significant positive correlation between the amount of HSP (hsp27 and -70) expression and patient survival has been also reported in esophageal carcinoma patients (Kawanishi et al., 1999). Patients with HSP-positive tumors can be expected to display a better prognosis than those with HSP-negative tumors.

We had previously identified that the expression of Hsp27 and Hsp40-like protein correlated with the invasive ability of lung cancer cell lines (Chen et al., 2001). In the present study, Hsp27, Hsp40-like protein, and Hsp70 were up-regulated by curcumin. Previous studies have also shown that curcumin could induce the heat shock response in vitro as measured by expression of Hsp70 in Hela cells (Dunsmore et al., 2001). The roles of these HSPs in curcumin-regulated tumor cell proliferation and invasion are still unclear and require additional study. These HSPs may have potential for anticancer therapeutic uses and are worthy of further investigation.

Several genes whose expression was not altered by curcumin in this study have been reported elsewhere to be involved in curcumin-regulated cell proliferation, apoptosis, or cancer invasion, including c-*jun*, c-*fos*, c-*myc*, bcl-2, *bcl*-xl, *p53*, *cyclin B*, *cyclin D1*, *COX-2*, and *MMP9* (Shishodia et al., 2003). It might be suggested that curcumin-regulated pathways were differed in different cell types. Previous study has also shown that curcumin-induced cell death and antiproliferation was caused neither by apoptosis nor by any significant change in the expression of apoptosis-related genes, including *bcl*-2, *p53*, *cyclin B*, and *transglutaminase* in human breast tumor cell lines (Mukhopadhyay et al., 2001). In the present study, TOPO-I, IIα, NF-κB, and heat shock proteins (Hsp27, -40, and -70) seem play the major roles in the anti-invasive and antiproliferative effects of curcumin in CL1–5 cells.

The results of this comprehensive survey demonstrated that curcumin exhibits antimetastatic activity in lung carcinoma cells. The anticancer potential of curcumin stems from its ability to suppress cancer cell proliferation and invasion by down-regulating the expression of MT1-MMP, NCAM, TOPO-I and II, and AXL-receptor tyrosin kinase and inhibiting the activity of MMP2 and the transcriptional activity of NF-κB. Conversely, the HSPs (Hsp27, Hsp40-like protein, and Hsp70) were up-regulated by curcmin. Nonetheless, their role in curcumin-regulated cell proliferation and invasion still requires additional evidence. The use of cDNA microarrays in this study has provided a global view of the cellular response of cancer cells to curcumin at genomic levels. Such global analysis of the anti-invasion response to the development of new novel drugs will require additional insight into the mechanisms of drug action for the treatment of cancer and allow for a more rational design of effective treatment strategies.

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