Metastatic genes targeted by an antioxidant in an established radiation- and estrogen-breast cancer model

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Received March 16, 2017; Accepted August 23, 2017

DOI: 10.3892/ijo.2017.4125

Abstract. Breast cancer remains the second most common disease worldwide. Radiotherapy, alone or in combination with chemotherapy, is widely used after surgery as a treatment for cancer with proven therapeutic efficacy manifested by reduced incidence of loco-regional and distant recurrences. However, clinical evidence indicates that relapses occurring after radiotherapy are associated with increased metastatic potential and poor prognosis in the breast. Among the anticarcinogenic and antiproliferative agents, curcumin is a well-known major dietary natural yellow pigment derived from the rhizome of the herb *Curcuma longa* (Zingiberaceae). The aim of the present study was to analyze the differential expression of metastatic genes in radiation- and estrogen-induced breast cancer cell model and the effect of curcumin on such metastatic genes in breast carcinogenesis. Expression levels of TGF-α and TGFβ1 genes were upregulated in MCF-10F and downregulated in Tumor2 cell lines treated with curcumin. Expression levels of other genes such as caspase 9 and collagen 4 A2 were upregulated in both MCF-10F and Tumor2-treated cell lines. Integrin α5 and cathepsin B and D decreased its expression in Tumor2, whereas E-Cadherin, c-myc and CD44 expressions were only increased in MCF-10F. It can be concluded that metastatic genes can be affected by curcumin in cancer progression and such substance can be used in breast cancer patients with advanced disease without side-effects commonly observed with therapeutic drugs.

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Abbreviations: IR, ionizing radiation; HBEC, human breast epithelial cell; LET, linear energy transfer

Key words: estrogen, radiation, metastatic microarray, breast cancer

Introduction

Breast cancer remains the second most common cancer worldwide with nearly 1.7 million new cases in 2012 (1). In cancer treatment, radiotherapy, alone or in a combination with chemotherapy, is widely used after surgery with proven therapeutic efficacy manifested by reduced incidence of loco-regional and distant recurrences (2-4). However, clinical evidence indicates that relapses occurring after radiotherapy are associated with increased metastatic potential and poor prognosis in breast (5,6) and other tissues (7,8). This has also been confirmed experimentally in tumors growing within a previously irradiated mammary tissue that is more invasive and metastasized (9-11).

Metastasis is a complex, multistep biological process, involving a multitude of genes and biomolecules. Despite the successful therapeutic management of breast cancer to control primary tumor growth, metastatic disease remains the greatest clinical challenge in oncology, as there are still not very efficient methods to prevent relapses and check the breast cancer metastasis. The interactions between cancer cells and normal host cells contribute significantly to the metastatic cascade, and a wide range of signaling and stimulating biomolecules and genes are involved in this process. Various authors have showed that heterogeneous nature of breast carcinomas that are not only characterized on the basis of histopathological features but can also be subdivided based on metastases gene-expression analysis (12-14).

Cancer metastases are responsible for the majority of cancer-related deaths. It usually arises from few cells in the primary tumor that acquire the ability to progress by sequential steps necessary to grow at a distant site (15,16). Some of these sequential steps include invasion through extracellular matrix, intravasation, survival in the circulation, extravasation into a distant site, and progressive growth at that site (16).

Although early-stage breast cancer is highly treatable, no effective treatment is available for metastatic breast cancer that follows surgery, radiation and chemotherapy for the primary tumor. In breast cancer, for example, metastasis affects the bone and the lung, and less frequently the liver, brain and adrenal medulla. Although, the genetic basis of these differential metastatic properties are poorly understood, acquisition of the ability to complete each step involved in metastasis is

thought to be driven by the accumulation of genetic mutations. These rare mutations are acquired at relatively late stage of the disease during the evolution of the primary tumor (17,18). Among the anticarcinogenic and antiproliferative agents, curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione;diferuloylmethane] is a well-known major dietary natural yellow pigment derived from the rhizome of the herb *Curcuma longa* (Zingiberaceae) (19). This phytochemical has also been shown to suppress the proliferation of numerous types of tumor cells (20,21). It has been previously shown to prevent the formation of many chemically-induced cancers including mammary cancer in mice (22,23). Metastatic development in human mammary epithelial carcinoma MCF-7 cells was also found to be inhibited by curcumin via the suppression of urokinase-type plasminogen activator by NF-κB signaling pathways (24). It is also a potent blocker of NF-κB activation induced by different inflammatory stimuli through inhibition of various cell cycle pathways, thus resulting in the suppression of NF-κB-dependent gene products that suppress apoptosis and mediate proliferation, invasion and angiogenesis (25-28). The human multidrug-resistant breast cancer cell line (MCF-7/TH) has been shown to be several-fold more sensitive to curcumin than the mammary epithelial cell line (MCF-10A). Even though both cell lines accumulated a similar amount of curcumin, a significantly higher percentage of apoptotic cells was induced in breast cancer cells compared to a very low percentage of apoptosis in mammary epithelial cells (29). The synergistic mechanisms of combinatorial treatment using curcumin and mitomycin C (MMC) on the inhibition of tumor growth were also explored by differential gene expression profile, Gene Ontology (GO), Ingenuity Pathway Analysis (IPA) and Signal-Net network analysis (30).

The development of DNA microarray technology of genome-wide transcriptomic profiling, has provided new insight into the genetic basis of metastasis The human tumor metastasis GE Array represents ~84 genes related to metastasis and it was utilized to study the effect of curcumin on radiation-induced breast cell model (31,32). Genes selected for this array encode several classes of growth factor receptors, cell-matrix interaction molecules, metastasis-associated proteases and suppressors, oncogenes and various signal transduction molecules. The aim of the present study was to analyze the differential expression of metastatic genes in radiation- and estrogen-induced breast cancer cell model and the effect of curcumin on such metastatic genes reported previously in breast carcinogenesis.

Materials and methods

Breast cancer cell lines. An *in vitro* experimental breast cancer model (Alpha model) developed by exposure of the immortalized human breast epithelial cell line, MCF-10F to low doses of high LET (linear energy transfer) α particle radiation (150 keV/μm) and subsequent growth in the presence or absence of 17β-estradiol was used in the present study (31). The spontaneously immortalized breast epithelial cell line MCF-10F has a near diploid karyotype and is of luminal epithelial cell origin. These cells retain all the characteristics of normal epithelium *in vitro*, including anchorage-dependence, non-invasiveness and non-tumorigenicity in the nude mice. Cell lines were grown in DMEM/F-12 (1:1) medium supplemented with antibiotics [100 U/mI penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B (all from Life Technologies, Grand Island, NY, USA)], 10 μg/ml and 5% equine serum (Biofluids, Rockville, MD, USA), 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO, USA) and 0.02 μg/ml epidermal growth factor (Collaborative Research, Bedford, MA, USA) (33). This model consisted of human breast epithelial cells in different stages of transformation: i) a control cell line, MCF-10F; ii) a malignant and tumorigenic cell line named Alpha5; and iii) Tumor2 derived from cells originated from a tumor after injection of Alpha5 cell line in the nude mice. The MCF-10F cell line was exposed to double doses of 60 cGy of α particles and treated with estrogen before the two exposures, such cell line was called Alpha5. Tumor2 cell line was originated from this cell line after injection in the nude mice. Both cell lines were treated with curcumin (30 μM for 48 h). Phenotypic characteristics of these cell lines and their genetic alterations including differentially expressed genes were previously described (31-36).

Isolation and purification of total RNA and mRNA. Total RNA was isolated from both the controls (MCF-10F) and Tumor2 cell lines and curcumin-treated cell lines using TRIzol reagent (Invitrogen Corp., Long Island, NY, USA). Each sample comprising 500 μg of total RNA was treated with 5 μl of DNAse I (10 U/μl) (Roche Pharm., Indianapolis, IN, USA) for 60 min at 37˚C. Then, 10X Termination Mix (0.1 M EDTA, pH 8.0 and 1 mg/ml glycogen) (Clontech, Palo Alto, CA, USA) was used to stop the reaction. Each sample was then purified following established procedure (32,37). The amount of each purified RNA sample was first measured by a spectrophotometer and then electrophoresed on denaturing formaldehyde/agarose/ethidium bromide gel, to check its quality and purity from proteins and free nucleotides. Each sample of 500 μg of purified total RNA was then subjected to PolyA$^+$ RNA analysis with the Oligotex mRNA Purification kit (Qiagen Inc., Valencia, CA, USA). PolyA$^+$ RNA was then purified following established procedures (37).

Development of cDNA from mRNA. A sample of 0.5-1 μg of PolyA$^+$ RNA was then used for First Strand cDNA Synthesis using the Advantage™ RT-for-PCR kit (Clontech) using oligo(dT) and random hexamer primers. Approximately 100 ng of the First Strand cDNA Synthesis product was used for carrying out RT-PCR reactions using gene specific primers as mentioned above. The PCR amplified products were then labeled using respective primers and Biotin-16-UTP as well as RT cocktail, as before, to generate the probes, and then used for cDNA hybridization analysis.

Protein expression by immunocytochemistry. Exponentially growing cells were plated on a glass chamber slide (Nunc Inc., Naperville, IL, USA), at a density of 1x10^4 cells/ml of medium, and allowed to grow until 70% confluence. Cells were fixed with buffered paraformaldehyde, incubated with 1% H_2O_2 in methanol to block endogenous peroxidase and washed with buffer solution, then covered with normal horse serum for 30 min, and then tested with mouse TGF-α (C-18; sc-1338), TGFβ1 (H-112; sc-7892), E-Cadherin (H-108; 7870) and

cathepsin D (E-7; sc-13148) monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 1:500 dilution overnight at 4˚C; then incubated for 45 min with diluted biotinylated secondary antibody solution and Vectastin Elite ABC reagent (both from Vector Laboratories, Burlingame, CA, USA). The experiments were repeated 3 times in cells with similar passages *in vitro*.

cDNA expression array. GE Array Q Series Human Tumor Metastasis Array (SABiosciences/Qiagen, Bethesda, MD, USA) represents 84 genes known to be involved in metastasis. Each of these genes was amplified by polymerase chain reaction (PCR) with gene-specific primers to generate 200- to 600-bp products. Each PCR product (~100 ng) that was spotted in quadruplicate onto a positively charged membrane. Each GEArray Q series membrane was spotted with a negative control of pUC18 DNA, blanks and housekeeping genes, including β-actin, GAPDH, cyclophilin A and ribosomal protein L13A (32).

Synthesis of cDNA probes from mRNA. The purified mRNAs were used for the synthesis of cDNA probes with Biotin-16-dUTP (Roche Pharm.). Annealing mixture was prepared by mixing ~1.0-5.0 μg of mRNA with 3 μl of Buffer A (GE primer mix) (SABiosciences) and the final volume was adjusted to 10 μl. The mixture was then incubated in a preheated thermal cycler at 70˚C for 3 min. Cooled to 42˚C and kept at that temperature for 2 min. Then, 10 μl of RT cocktail was prepared by mixing 4 μl of 5X Buffer BN [for 50 μl 10X buffer, add 1 μl of 1 M DTT and 50 μl of 10X dNTP mix (5 mM dATP, dCTP, dGTP and 500 μM dTTP)], 2 μl of Biotin-16-UTP, 2 μl of RNase-free H_2O, 1 μl of RNase inhibitor and 1 μl of MMLV reverse transcriptase (both from Promega Corp., Madison, WI, USA). RT cocktail was then warmed at 42˚C for 1 min and slowly mixed with 10 μl of pre-warmed annealing mixture. The incubation continued at 42˚C for 90 min, and then labeled cDNA probe was denatured by heating at 94˚C for 5 min, and quickly chilled on ice. In each cell line tested, mRNA was isolated and purified from different passages, and cDNA probes were prepared from each of them and hybridized to the respective membranes. Experiments using the same mRNA preparation were repeated 3 times, and measurable, median-normalized expression values of each gene were compared to avoid false-positive signals (32).

Differential hybridization of cDNA expression array. Each array membrane was pre-wetted with 5 ml of de-ionized water and incubated at 60˚C for 5 min. It was then replaced with 2 ml of pre-warm (60˚C) GEAprehyb solution (GEAhyb solution with a heat-denatured sheared salmon sperm DNA at a final concentration of 100 μg/ml) (SABiosciences, Bethesda, MD) and gently mixed for a few seconds. Pre-hybridization was continued at 60˚C for 1-2 h with continuous gentle agitation. Approximately 0.75 ml solution of GEAhyb was prepared by adding the entire volume of denatured cDNA probe onto GEAprehyb solution and kept at 60˚C. Then, GEAprehyb solution was replaced by GEAhyb solution and hybridization continued overnight at 60˚C with continuous gentle agitation. Subsequently, array membranes were washed twice in wash solution 1 (2X sodium chloride sodium citrate and 1% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation and then twice with solution 2 (0.1X sodium chloride sodium citrate and 0.5% sodium dodecyl sulfate) at 60˚C for 15 min each with gentle agitation. To assess the reproducibility of the hybridization array assays, pair-wise comparisons between array data sets for each cell line was tested by repeated hybridization and the mRNAs prepared in different lots were analyzed in scatter plots with multiple regressions (32,38). In each case, expression levels of 95% of the genes had repeated values that were within 2-fold (32).

Chemiluminescent detection of cDNA probes. After discarding the last wash, 2 ml of GEAblocking solution was added to each membrane and incubated for 40 min at room temperature with continuous agitation. Then, binding buffer was prepared by diluting alkaline phosphatase-conjugated streptavidin (AP) with 1X buffer F (SuperArray, Bethesda, MD, USA) in a 1:7,500 dilution. GEAblocking solution was replaced by 2 ml of binding buffer and incubated for 10 min with continuous but gentle agitation. Then, membrane was washed 4 times with 4 ml of 1X binding buffer F for 5 min in each washing and rinse twice with 3 ml of rinsing buffer G (SuperArray). The membrane was covered with 1.0 ml of CDP-Star chemiluminescent substrate and incubated at room temperature for 2-5 min. It was then exposed to X-ray film (Kodak BioMax MS Film; Kodak Corp., Rochester, NY, USA) with corresponding intensifying screen at room temperature for multiple exposures of 1-5 min.

Quantification of array hybridization. Quantification of hybridization signals on the expression array membranes was carried out by exposing the autoradiographic film in a densitometric scanner (model 300A; Molecular Dynamics, Sunnyvale, CA, USA). It was then estimated both with the ImageQuant (Molecular Dynamics) and ScanAlyze program (Eisen Lab). Volume quantification was performed by calculating the volume under the surface created by a 3-dimensional plot of pixel locations and pixel values as previously described (32,38). All raw signal intensities were corrected for background by subtracting the signal intensity of a negative control or blank. Results were also normalized to that of a housekeeping gene. These corrected, normalized signals can then be used to estimate the relative abundance of particular transcripts. To delineate the potential signal interference between adjacent strong hybridization signals, equal-sized ellipses were drawn around each signal area (hybridization spots) using software (ImageQuant/ScanAlyze) and was then separately scanned and compared with housekeeping genes so the chances of interference between adjacent strong hybridization signals were minimized. Normalization of the expression levels of different housekeeping genes from multiple autoradiographic exposures between different hybridization experiments were carried out by taking the average signals of each of the housekeeping genes. Data from high intensity spots were chosen for further use. Median background was subtracted, and signals that were <2.0-fold above background level were considered too low to accurately measure and were omitted from the analysis. Signals for each individual gene were also normalized to the geometric mean of the expression level of that gene across the set of membranes being compared.

Table I. GEArray Q series of human tumor metastasis gene array.

Functional gene grouping	Gene symbols/names
Growth factors and receptors	CSF1 (csf-1), CSF1R (c-fms/MC-SF-R), FGF1 (a-FGF), FGF2 (b-FGF2), HGF (scatter factor), IGF2, NGFB, TGFA (TGF-α), TGFB1 (TGFβ1), VEGF, VEGFC
Cell-cell and cell-matrix interaction molecules	CAV1 (caveolin-1), CDH1 (cadherin-1/E-cadherin), COL4A2 [collagen α2 (IV)], ICAM5 (telencephalin), ITGA2-3, ITGA5-6, ITGA6, ITGB1, ITGB3, LAMB1 (laminin β1), LAMC1 (laminin β2), MICA (MUC-18), MUC1, NCAM1, PECAM1, VTN (vitronectin)
Metastasis-associated proteases: Matrix metalloproteinases	MMP1-3, MMP7-11, MMP13-16
Others	CASP8-9, CST3 (cystatin C), CTSB (Cathepsin B), CTSD (cathepsin D), CTSL (cathepsin L), ELA2 (elastase), HPSE (heparanase), MGEA5 (meningioma hyaluronidase 5), PLAU (uPA), TMPRSS4
Protease inhibitors	SERPINB2 (PaI-2), SERPINB5 (maspin), SARPINE1 (PAI-1), THBS1-S2, TIMP1-P3
Signal transduction molecules	LIMK1 (Lim kinase), PLAUR (uPAR), PIK3C2B, RAC1
Oncogenes	ERBB2 (c-erb-2/neu), ETS1-2 (c-ets-1-2), ETV4 (PEA3), FES, FOS (c-fos), HRAS (c-Ha-ras), MDM2, MYC (c-myc), RAF1, SRC (c-src)
Metastasis, suppressors	BRMS1 (BrMS1), CD44, DCC, KAI1, KISS1 (KiSS-1), MAP2K4 [mkk4 (JNKK1)], MTA1, NM23A (NM23), NME4, PTEN
Other related genes	API5 (apoptosis inhibitor 5), ARHC (Rho C), EHM2, ENPP2 (autotaxin/ATX), MGAT3 and 5 (acetylglucosa-minyltransferase III and V), ODC1, PTGS2 (cox-2), S100A4 (mts-1), SNCG (BCSG1), SSP1 (osteopontin)

Mean signals were calculated from quadruplicate measurable spots, or whether 3 of the 4 spots were measurable. Then, the changed folds indicated whether a gene exhibited increased, decreased, or unchanged expression based on statistical criteria (38).

Western blot analysis. Differential expression of few genes after quantification by array were further confirmed by western blot analysis following usual procedures.

Results

The effect of curcumin on cell lines from a radiation-induced breast cell model was studied. Microarray technology allowed us to measure the relative expression of many genes in a single experiment. In the present study, two controls the MCF-10F and Tumor2 cell lines were treated with curcumin (MCF-10F+curcumin and Tumor2+curcumin). Table I shows the genes selected for this array that encode several classes of growth factor receptors, cell-cell, cell-matrix interaction molecules, metastasis-associated proteinase, protease inhibitors, signal transduction molecules oncogenes, metastasis, suppressors and other related genes. Fig. 1 corresponds to the membranes containing the genes found in this particular array as GE Array Q Series Human Tumor Metastasis Gene Array that was used.

Results indicated that out of 84 genes, 16 were upregulated and 4 were downregulated by curcumin treatment in MCF-10F cell line, whereas, a total of 24 genes were altered with 11 upregulated and 13 downregulated by curcumin treatment in Tumor2 cell line. In both cases, alterations of Q ≥2.0 were taken into consideration whereas, ~20-25 genes also showed moderate alterations in their expression (Q <1.50) (data not shown). The remaining 30-35 genes showed no altered expression in either group of experiments.

Table II A and B show upregulated and downregulated metastatic genes found in MCF-10F control and treated with curcumin cell lines, respectively. Fig. 2 presents differentially expressed genes found in such array, where a comparison between MCF-10F and MCF-10F plus curcumin cell lines were analyzed. Table III A and B show regulated and downregulated metastatic genes found in Tumor2 cell line control and treated with curcumin cell lines. Fig. 3 presents differentially expressed genes found in such array, where a comparison between Tumor2 and Tumor2 plus curcumin cell lines were analyzed. Protein expression of growth factors such as TGF-α (Fig. 4A) was upregulated in MCF-10F cells treated with curcumin. However, it was downregulated in Tumor2 by this substance (Fig. 4B). Similar results were observed in TGFβ1 protein expression in Tumor2 cell lines treated with curcumin (Fig. 4C and D). Among other important genes, E-Cadherin protein expression was upregulated in MCF-10

Table II. Upregulated and downregulated metastatic genes in MCF-10F cell line treated with curcumin (30 μM).

A, Upregulated metastatic genes in MCF-10F cell line treated with curcumin (30 μM)

Position	UniGene	GeneBank	Symbol	Description	Gene name	Fold change	
						MCF-10F	MCF-10F+cur
56	Hs.90800	D83646	MMP16	Matrix metalloproteinase 16 (membrane-inserted)	MMP16	-	4.7 (↑)
86	Hs.170009	NM_003236	TGFA	Transforming growth factor, α	TGF-α	-	4.6 (↑)
87	Hs.1103	X02812	TGFB1	Transforming growth factor, β1	TGFβ1	-	4.5 (↑)
94	Hs.73793	M32977	VEGF	Vascular endothelial growth factor	VEGF	-	3.9 (↑)
8	Hs.194657	Z13009	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	E-Cadherin	-	3.6 (↑)
49	Hs.90598	NM_000247	MICA	*Homo sapiens* MICA gene, allele MUC-18	MUC-18	-	3.6 (↑)
64	Hs.79070	X00364	MYC	v-myc avian myelocytomatosis viral oncogene homolog	c-myc	-	3.6 (↑)
95	Hs.79141	X94216	VEGFC	Vascular endothelial growth factor-C	VEGF-C	-	3.4 (↑)
47	Hs.121502	NM_002410	MGAT5	cDNA encod N-acetylglucosamyltransferase-V (*Homo sapiens*)	GnT-V	-	3.1 (↑)
91	Hs.325495	NM_003255	TIMP2	Tissue inhibitor of metalloproteinase 2	TIMP-2	-	2.8 (↑)
16	Hs.211567	NM_005215	DCC	Deleted in colorectal carcinoma	DCC	-	2.5 (↑)
71	Hs.78146	NM_000442	PECAM1	*Homo sapiens* platelet endothelial cell adhesion molecule (CD31 ag)	CD31	-	2.3 (↑)
5	Hs.100641	U60521	CASP9	Caspase 9, apoptosis-related cysteine protease	Caspase 9, Mch6	-	2.1 (↑)
9	Hs.75617	X05610	COL4A2	Collagen, type IV, α2	Collagen 4 A2	-	2.0 (↑)
7	Hs.169610	M59040	CD44	CD44 antigen (homing function and Indian blood group system	CD44	-	2.0 (↑)
51	Hs.2258	NM_002425	MMP10	Matrix metalloproteinase 10	Stromelysin 2	-	2.0 (↑)

B, Downregulated metastatic genes in MCF-10F cell line treated with curcumin (30 μM)

Position	UniGene	GeneBank	Symbol	Description	Gene name	Fold change	
						MCF-10F	MCF-10F+cur
93	Hs.63325	NM_019894	TMPRSS4	Transmembrane protease, serine 4	TMPRSS-4	-	5.3 (↓)
78	Hs.85181	X03484	RAF1	v-raf-1 murine leukemia viral oncogene homolog 1	c-raf-1	-	4.7 (↓)
34	Hs.265829	M59911	ITGA3	Integrin, α3 (ag CD49C, α3 sub of VLA3 rec.)	Integrin α3	-	4.0 (↓)
31	Hs.151250	U72671	ICAM5	Intercellular adhesion molecule 5, telencephalin	ICAM-5	-	3.4 (↓)
97-99	N/A	L08752	PUC18	PUC18 plasmid DNA	pUC18	1.0	1.0
100-102	Blank	Blank	Blank	Blank	0	0.0	0.0
103-104	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.0	1.0
105-108	Hs.342389	NM_021130	PPIA	*Homo sapiens* peptidylprolyl isomerase A	Cyclophilin A	1.0	1.0
109-110	Hs.119122	NM_012423	RPL13A	Ribosom. protein L13a (23 kDa highly basic protein)	RPL13A	1.0	1.0
111-112	Hs.288061	X00351	ACTB	β-actin	β-actin	1.0	1.0

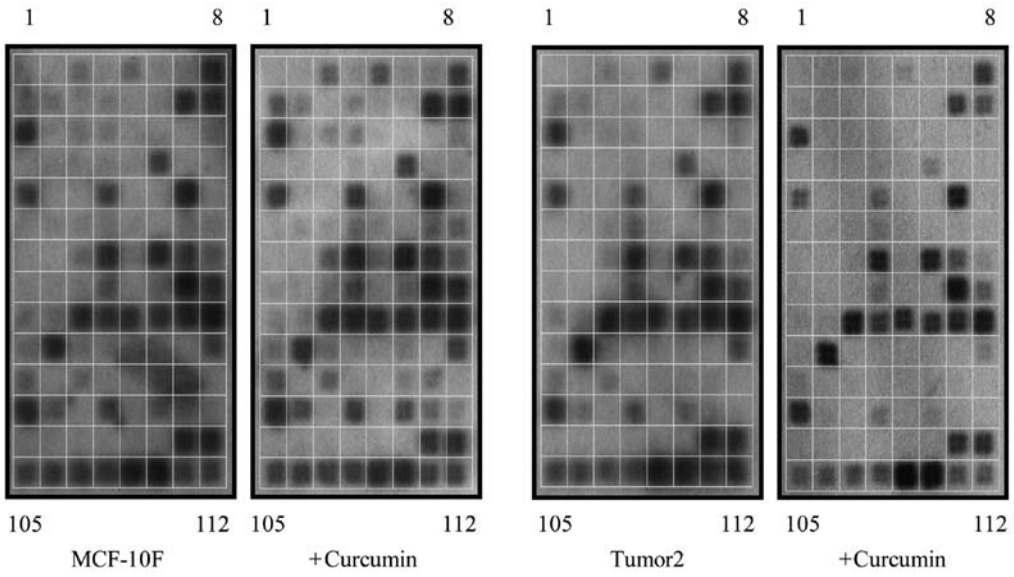

Figure 1. GE Array Q series of human tumor metastasis gene array: Differentially expressed genes of a human tumor metastasis gene array with MCF-10F and Tumor2 cell lines treated with curcumin, respectively.

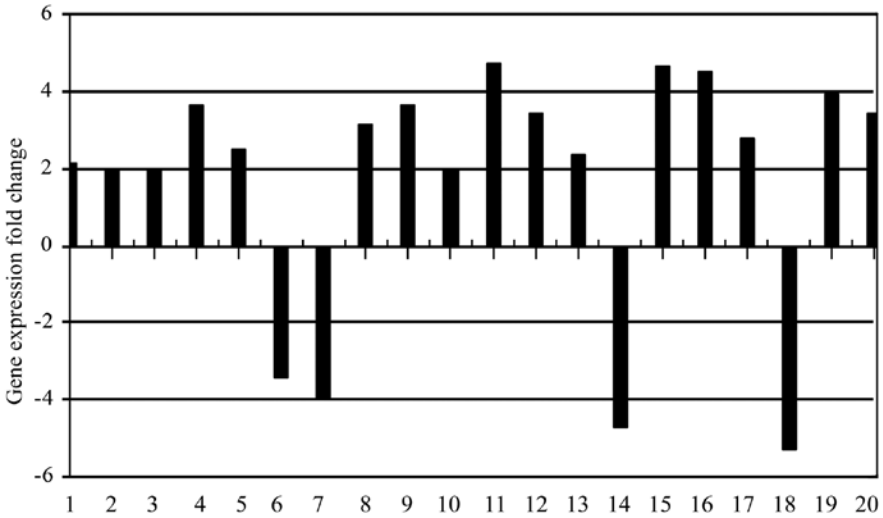

Figure 2. Graph that represents the differentially expressed genes from a human tumor metastasis gene array: a comparison between MCF-10F and MCF-10F plus curcumin cell lines.

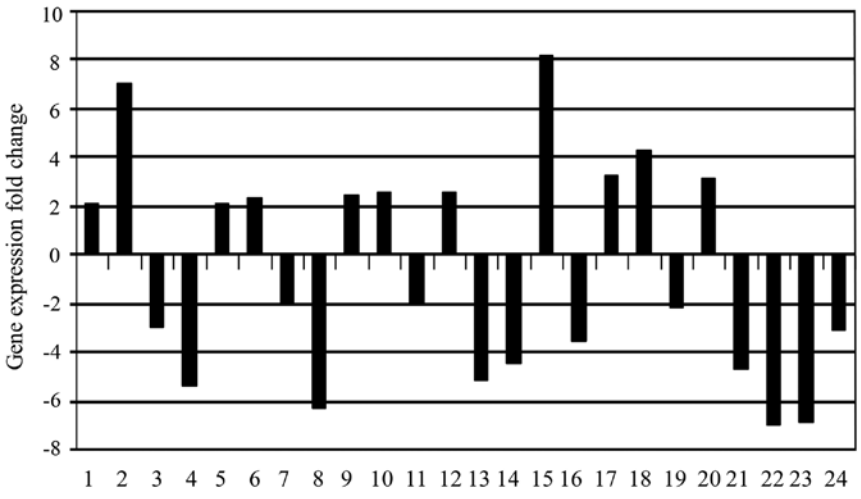

Figure 3. Graph that represents the differentially expressed genes in a human tumor metastasis gene array: a comparison between Tumor2 and Tumor2 plus curcumin cell lines.

Table III. Upregulated and downregulated metastatic genes: Tumor2 cell line treated with curcumin (30 μM).

A, Upregulated metastatic genes: Tumor2 cell line treated with curcumin (30 μM)

Position	UniGene	GeneBank	Symbol	Description	Gene name	Fold change	
						Tumor2	Tumor2+cur
60	Hs.73862	NM_002424	MMP8	Matrix metalloproteinase 8 (neutrophil collagenase)	MMP8	-	8.2 (↑)
9	Hs.75617	X05610	COL4A2	Collagen, type IV, α2	Collagen 4 A2	-	7.0 (↑)
76	Hs.196384	NM_000963	PTGS2	*Homo sapiens* prostaglandin-endoperoxide synthase 2 (prostaglandin G/hsynthase and cyclooxygenase)	Cox-2	-	4.3(↑)
75	Hs.10712	U96180	PTEN	Phosphatase and tensin homolog (mut in multiple Adv cancers 1)	PTEN	-	3.2 (↑)
80	Hs.75716	J02685	SERPINB2	Human plasminogen activator inhibitor	PAI-2	-	3.1(↑)
30	Hs.37003	NM_005343	HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog	H-ras	-	2.5 (↑)
36	Hs.227730	X53586	ITGA6	Integrin, α6 subunit	Integrin α6	-	2.5 (↑)
28	Hs.809	X57574	HGF	Hepatocyte growth factor) (hepapoietin A; scatter factor	Scatter factor	-	2.4 (↑)
24	Hs.7636	X52192	FES	Proto-oncogene tyrosine-protein kinase fes/fps	c-fes	-	2.3 (↑)
20	Hs.323910	m11730	ERBB2	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2	erb-2	-	2.1 (↑)
5	Hs.100641	U60521	CASP9	Caspase 9, apoptosis-related cysteine protease	Caspase 9, Mch6	-	2.1 (↑)

B, Downregulated metastatic genes: Tumor2 cell line treated with curcumin (30 μM)

Position	UniGene	GeneBank	Symbol	Description	Gene name	Fold change	
						Tumor2	Tumor2+cur
86	Hs.170009	NM_003236	TGFA	Transforming growth factor, α	TGF-α	-	7.0 (↓)
87	Hs.1103	X02812	TGFB1	Transforming growth factor, β1	TGFβ1	-	6.9 (↓)
27	Hs.25647	V01512	FOS	Human cellular oncogene c-fos	c-fos	-	6.3 (↓)
14	Hs.343475	M11233	CTSD	Cathepsin D (lysosomal aspartyl protease)	Cathepsin D	-	5.4 (↓)
51	Hs.2258	NM_002425	MMP10	Matrix metalloproteinase 10	Stromelysin 2	-	5.1 (↓)
83	Hs.63236	NM_003087	SNCG	Synuclein, γ (breast cancer-specific protein 1)	BCSG1	-	4.7 (↓)
57	Hs.111301	J03210	MMP2	Matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase)	Gelatinase A	-	4.5 (↓)
66	Hs.2561	X52599	NGFB	Nerve growth factor, β polypeptide	NGFβ	-	3.5 (↓)
95	Hs.79141	X94216	VEGFC	Vascular endothelial growth factor-C	VEGF-C	-	3.1 (↓)
13	Hs.297939	L16510	CTSB	Cathepsin B	Cathepsin B	-	3.0 (↓)
26	Hs.284244	NM_002006	FGF2	Fibroblast growth factor 2 (basic)	β-FGF	-	2.0 (↓)
35	Hs.149609	X06256	ITGA5	Integrin, α5 (fibronectin receptor, α polypeptid)	Integrin α5	-	2.0 (↓)

Table III. Continued.

B, Downregulated metastatic genes: Tumor2 cell line treated with curcumin (30 μM)

Position	UniGene	GeneBank	Symbol	Description	Gene name	Fold change	
						Tumor2	Tumor2+cur
77	Hs.173737	NM_006908	RAC1	Ras-rel C3 bot. toxin subs. 1 (rho family, small GTP bind prot.)	Rac1	-	2.0 (↓)
97-99	N/A	L08752	PUC18	PUC18 Plasmid DNA	pUC18	1.0	1.0
100-102	Blank	Blank	Blank	Blank	0	0.0	0.0
103-104	Hs.169476	M33197	GAPD	Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.0	1.0
105-108	Hs.342389	NM_021130	PPIA	*Homo sapiens* peptidylprolyl isomerase A	Cyclophilin A	1.0	1.0
109-110	Hs.119122	NM_012423	RPL13A	Ribosom protein L13a (23 kDa highly basic protein)	RPL13A	1.0	1.0
111-112	Hs.288061	X00351	ACTB	β-actin	β-actin	1.0	1.0

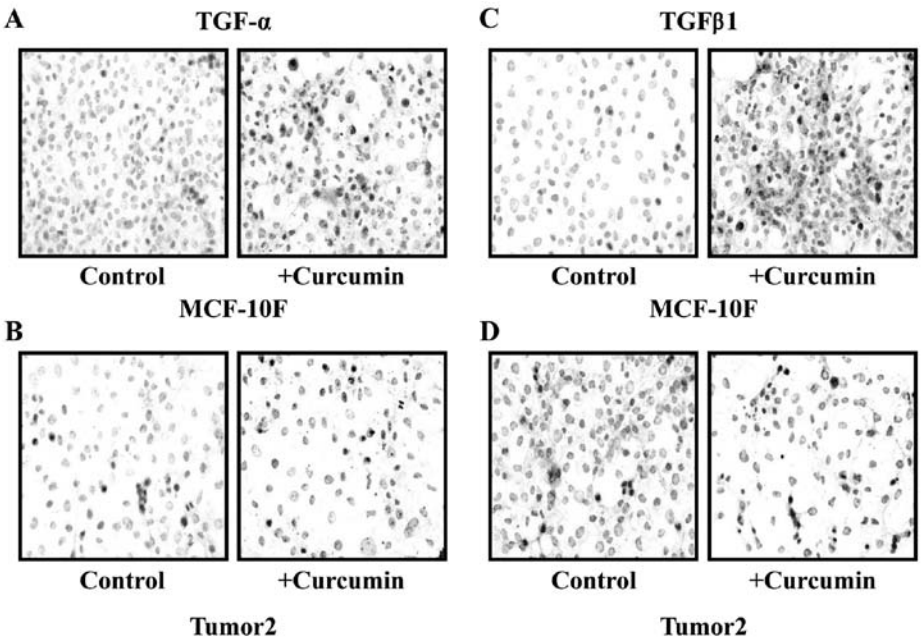

Figure 4. Representative images of protein expression of transforming growth factor-α (TGF-α) in: (A) MCF-10F control; (B) treated with curcumin cell lines; (C) Tumor2 and (D) treated with curcumin cell lines, respectively.

cells by the effect of curcumin and no effect in the Tumor2 cell line as shown in Fig. 5A and B). However, cathepsin D protein expression was not affected by curcumin in the MCF-10F cell line and decreased such expression in the Tumor2 cell line as seen in Fig. 5C and D. The expression levels of few selected genes identified by cDNA microarray expression profiling were further validated by western blot analysis (Fig. 6).

Discussion

Microarray technology allows us to measure the relative expression of thousands of genes in a single experiment. Advancement in microarray technology and gene expression databases provide a new opportunity for identifying the mode of action and targets for various genes involved in breast cancer metastatic cascade. As breast cancer is one of the most common and complex types of cancer, which frequently progress towards metastasis, microarray technique is ideal to study the genes associated with this process.

The aim of the present study was to investigate the effect of antiproliferative compound curcumin in a radiation-induced breast cancer model on genes involved in breast cancer metastasis. In the present study, the two controls MCF-10F and Tumor2 cell lines, were used and both were treated with 30 mM

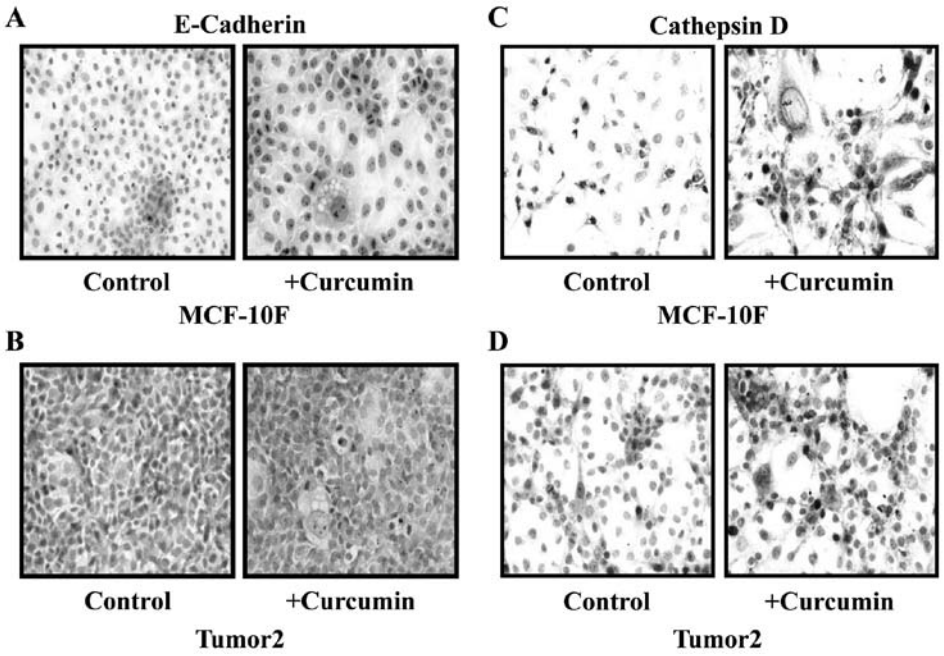

Figure 5. Representative images of protein expression of transforming growth factor β (TGFβ) in: (A) MCF-10F control; (B) treated with curcumin cell lines; (C) Tumor2 and (D) treated with curcumin cell lines, respectively.

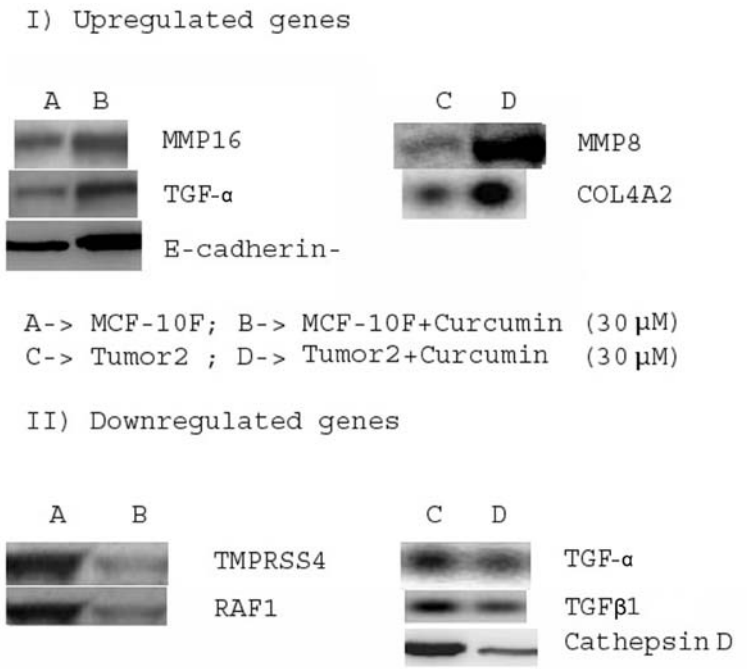

Figure 6. Confirmation of differential gene expression of few genes selected from human tumor metastasis gene array in radiation- and curcumin-treated breast cell model by western blotting.

curcumin as MCF-10F+curcumin and Tumor2+curcumin cell lines.

Various genes related to metastasis were differentially altered in the human metastasis gene array after cell lines were treated with curcumin. Thus, curcumin showed upregulation of TGF-α expression in MCF-10F cell line but downregulated in Tumor2 cell line. This result is consistent with the role of TGF-α during brain metastases where estrogen was found to promote colonization of triple-negative breast cancer cells by upregulation of TGF-α mRNA and protein levels via astrocytes mediated paracrine mechanism which activated EGFR in brain metastatic cells (39). Similar results were obtained when TGFβ1 expression was analyzed. Various curcuminoids are known to block TGFβ1 signaling in human breast cancer cells and limit osteolysis in a murine model of breast cancer bone metastasis which is corroborated with these findings (40).

Results also indicated that another important gene, the vascular endothelial growth factor-C (VEGF-C) altered the

expression since it was upregulated in MCF-10F cell line and downregulated in Tumor2 cell line in presence of curcumin. In a xenograft model of triple-negative breast cancer in mice, a lower expression of VEGFR2/3 and inhibition of angiogenesis was noted in presence of curcumin. Curcumin is known to inhibit VEGF-C induced lymphangiogenesis in a matrigel assay in mice, which is fully consistent with the result obtained in curcumin treated Tumor2 cell line (41,42). Flow cytometry and western blot analysis showed that curcumin and its derivatives induced cell cycle arrest at G_0/G_1 phase in MCF-7 cells by entering early phase of apoptosis via mitochondrial pathway, as evidenced by the activation of caspase 3 and 9, which led to elevation of intracellular ROS, a decrease in Bcl-2 and PARP and as well as an increase in Bax expression (43).

Other genes as caspase 9 and collagen 4 A2 were upregulated in both MCF-10F and Tumor2 groups. Angiogenesis is a crucial step in the growth and metastasis of cancers and the role of curcumin to act as an angiogenesis inhibitor by modulating collagen-like protease activity during endothelial morphogenesis and in few other systems are already established (44).

Several integrins (e.g., integrin $\alpha 3$, $\alpha 5$ and $\alpha 6$) showed differential expression in both cell lines. The downregulation of integrin-mediated signal transduction by curcumin treatment in Tumor2 cell line strongly indicated the potential role of curcumin as an inhibitor of tumor cell invasion and metastasis (45). Integrins are usually cell surface markers to detect various cancer stem cell activities. Differential gene alterations of different types of integrins were noted in the analysis of the present results. It has been reported that cancer stem cells resist conventional cancer therapies and are likely to play a major role in cancer relapse by upregulating the surface markers such as integrins that leads to metastasis (46).

Other important genes usually altered in cancer progression of breast were cathepsin B and E, members of papain family of cysteine proteases normally present in the lysosome. They can be translocated and are able to degrade components of the extracellular matrix. It is interesting to note that curcumin lower protein expression of both these genes in Tumor2 cell lines. It has been shown that an example of substrate for cathepsin B is E-Cadherin, which is involved in adherens junctions, where the downregulation of E-Cadherin in cancer is directly linked to invasion and metastasis (47,48). E-Cadherin expression was only upregulated in the MCF-10F, but not in the Tumor2 cell lines by the effect of curcumin. It is notable that such gene is related to epithelium mesenchymal transition, corroborating the epithelial phenotype that characterized E-Cadherin in such process (49).

It is concluded that metastatic genes can be affected by curcumin in cancer progression therefore it is interesting to determine that this substance can be used in breast cancer patients with advanced disease without side-effects usually induced by therapeutic drugs. Due to complex structure involving multiple functional groups, the exact mechanism and site of action of curcumin on breast cancer cells is difficult to determine and needs to be further studied.

Acknowledgements

In the present study, the technical support of Guiliana Rojas, Georgina Vargas Marchant and Leodán A Crispin is greatly appreciated. The present study was supported by grant support FONDECYT #1120006b (to G.M.C.) and Universidad de Tarapaca-Ministerio de Educación (MINEDUC); COBI Innovation-HCC (Title-V) (to D.R.).

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