RESEARCH ARTICLE

Genomic Screening for Targets Regulated by Berberine in Breast Cancer Cells

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Abstract

Berberine, a common isoquinoline alkaloid, has been shown to possess anti-cancer activities. However, the underlying molecular mechanisms are still not completely understood. In the current study, we investigated the effects of berberine on cell growth, colony formation, cell cycle distribution, and whether it improved the anticancer efficiency of cisplatin and doxorubicin in human breast cancer estrogen receptor positive (ER+) MCF-7 cells and estrogen receptor negative (ER-) MDA-MB-231 cells. Notably, berberine treatment significantly inhibited cell growth and colony formation in the two cell lines, berberine in combination with cisplatin exerting synergistic growth inhibitory effects. Accompanied by decreased growth, berberine induced G1 phase arrest in MCF-7 but not MDA-MB-231 cells. To provide a more detailed understanding of the mechanisms of action of berberine, we performed genome-wide expression profiling of berberine-treated cells using cDNA microarrays. This revealed that there were 3,397 and 2,706 genes regulated by berberine in MCF-7 and MDA-MB-231 cells, respectively. Fene oncology (GO) analysis identified that many of the target genes were involved in regulation of the cell cycle, cell migration, apoptosis, and drug responses. To confirm the microarray data, qPCR analysis was conducted for 10 selected genes based on previously reported associations with breast cancer and GO analysis. In conclusion, berberine exhibits inhibitory effects on breast cancer cells proliferation, which is likely mediated by alteration of gene expression profiles.

Keywords: Berberine - breast cancer - microarray - gene expression profile

Introduction

Breast cancer is one of the most common cancers and a leading cause of cancer mortality among women, and it accounted for approximately 23% of total new cases and 14% of all cancer deaths in 2008 (Jemal et al., 2011). Chemotherapy plays a major role in the breast cancer therapy. However, the most of the breast cancer patients eventually develop drug resistance and experience severe side effects. Therefore, it is imperative to develop innovative therapies to improve treatment outcomes for breast cancer patients. The use of natural herbal may find a new way for breast cancer treatment.

Berberine, a natural isoquinoline alkaloid derived from Berberis species, has been shown to possess anti-cancer activity and induces apoptosis in various cancer cell lines in a concentration-dependent manner (Tan et al., 2006; Meeran et al., 2008; Wu et al., 2012). Several mechanisms involved in the anti-cancer effects of berberine have been identified, which include inhibiting metastasis by decreased expression of MMPs, suppressing migration by reducing the expression of COX-2, PEG2 and its

receptor, inhibiting cancer cells growth and proliferation by induction of cell cycle arrest (Kim et al., 2008a; Kim et al., 2008b; Singh et al., 2011). Recent researches suggested that berberine induced cell cycle arrest in both breast cancer estrogen receptor positive and negative cells (Kim et al., 2008a). In MDA-MB-231 breast cancer cells, berberine inhibited the TNF-α-induced MMP9 expression and suppressed the activities of MMP2 and MMP9 (Kim et al., 2008b). In MCF-7 cells, berberine markedly inhibited cell proliferation and induced cell death of MCF-7 cells via a mitochondria and a caspase dependent apoptotic pathway (Patil et al., 2010). The combined treatment of MCF-7 and MDA-MB-231 cells with berberine and ER antagonists exerted an inhibitory effect on cell growth in MCF-7 cell but not in MDA-MB-231 cells (Liu et al., 2009). The findings of these reports suggested that mechanisms underlying berberine's growth inhibitory effects are different among cancer cell lines. Differential expression of the ER is likely to mediated drug responses in various pathways, and understanding the underlying mechanism is important in the treatment of the breast cancers.

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To better understand the physiological actions and the molecular targets of berberine in breast cancer cells, we used an oligonucleotide microarray which contained approximately 4×44k genes and undertook a gene expression profiling study to monitor the changes in the gene expression associated with sensitivities to berberine in ER+ MCF-7 and ER- MDA-MB-231 cell lines. This study was conducted to understand the molecular actions of berberine in breast cancer cell lines. Alteration of gene expression patterns may account for the inhibitory activity of berberine on breast cancer cell lines.

Materials and Methods

Materials

Berberine (>95% of purity; Sigma, St. Louis, MO) and cisplatin (Sigma) was dissolved in dimethylsulfoxide (DMSO; Sigma). Doxorubicin was purchased from Main Luck Pharmaceuticals Inc (Shenzhen, China) and dissolved in culture medium; the final concentration of the vehicle should not exceed 0.1%.

Cell culture

Human breast cancer MCF-7 and MDA-MB-231 cell lines were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were grown in RPMI 1640 medium (Hyclone, South Logan, UT) with 10% fetal calf serum (FBS; HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were sub-cultured twice weekly.

MTS assay

The cell viability was determined by MTS ((Promega, Madison, WI) assay (Sekhon et al., 2008). Briefly, 5×10³ cells per well were seeded in 96-well culture plates in triplicate and allowed to attach overnight. Then cells were exposed to different doses of drugs for 48h. After the treatment, 20 μl of MTS was added to each well and incubated for 4h at 37°C. The absorbance of each well was determined with the measurement wavelength of 490nm.

Colony formation analysis

The cells were treated with DMSO vehicle or 20 μM berberine for 48 h. Cells were then digested to reconstitute the single-cell suspension and seeded into 6-well plate (400 cells per well). Cells were incubated at 37°C and in an atmosphere of 5% CO_2. Two weeks later, the colonies were fixed with 100% methanol for 15 min and stained with crystal violet for 20 min then washed with phosphate buffered solution (PBS). Colonies were counted after taking photographs. The colony formation rate was calculated as number of colonies in berberine-treated/number of colonies of DMSO control ×100%. Each experiment was repeated at least three times.

Cell cycle analysis

Cells were harvested after DMSO or 20 μM berberine treatment for 48h, fixed in 70% cold ethanol at -20°C overnight. The fixed cells were wash with PBS and then resuspended in 300 μl PBS containing 0.5 mg/ml of propidium iodide (PI; eBioscience, San Diego, CA) and 0.1 mg/ml of Rnase A (eBioscience), then incubated at 37°C for 30 min. Cell cycle distribution was determined by Cytomics FC 500 flow cytometer (Beckman Coulter, CA).

Total RNA isolation and microarray analysis

MCF-7 and MDA-MB-231 cells were treated with 20μM berberine or DMSO for 48h, Total RNA from the treated and control cells were isolated by using Trizol (Invitrogen Valenica, CA, USA). The quality of all RNA was assessed by Agilent Bioanalyzer 2100 (Clara, CA, USA) Qualified total RNA was further purified by RNeasy mini kit (QIGEN, GmBH, Germany) and RNase-Free DNase set (QIGEN). Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Agilent, CA, US), following the instructions. Labeled cRNA were purified by RNase mini kit. 1.65μg Cy-labeled cRNA were hybridized and processed on an Agilent Human Whole Genomic 4×44K Oligo Microarray according to the manufacturer's instructions. After 17 hours hybridization, slides were washed in staining dishes (Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Agilent) according to the instructions. Slides were scanned by Agilent Microarray Acanner. Data were extracted with Feature Extraction software 10.7 (Agilent). Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent).

Real-time PCR verification

Sample (2 μg) of RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo) to prepare first strand cDNA. qPCR amplification was carried out using SYBR-green detection of PCR products in real time with an ABI-7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR program was initiated by 30s at 95°C before 40 thermal cycles, each of 5s at 95°C, 30s at 55°C, and 34s at 72°C. Data were analyzed according to the $2^{-\triangle\triangle Ct}$ method (Livak et al., 2001). β-actin gene was used as an endogenous control to normalize the expression of the samples.

Statistical analysis

The data were expressed as mean ± standard deviation. The results were analyzed using the one-way analysis of variance (ANOVA) test for over three groups or unpaired t-test for two groups and combinations using GraphPad Prism 4 software package (San Diego, CA, USA). A *p* value <0.05 was considered statistically significant.

Results

Berberine significantly inhibits MCF-7 and MDA-MB-231 cell growth

MCF-7 and MDA-MB-231 cells were treated with vehicle or increasing concentrations of berberine for 48h and the cytotoxicity of berberine measured by an MTS assay. As shown in Figure 1, berberine reduced the viability of MCF-7 and MDA-MB-231 cells in a dose-dependent manner. The IC_{50} values of berberine for MCF-7 and MDA-MB-231 cells were approximately 106 μM and
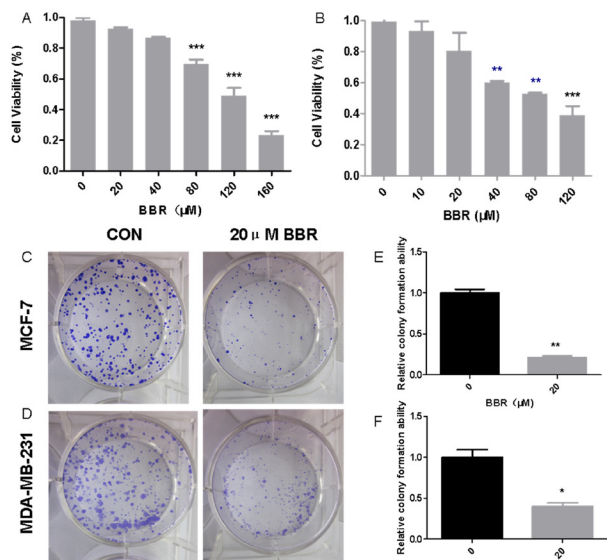
Figure 1. Growth Inhibition Effect of Berberine on MCF-7 and MDA-MB-231 Cells *in vitro*. Berberine reduces the viability of human breast cancer MCF-7 (A) and MDA-MB-231 (B) cells in a dose-dependent manner. Representative photographs of colony formation assay in MCF-7 (C) and MDA-MB-231 (D) cells. Quantification of the colony formation efficiency in MCF-7 (E) and MDA-MB-231 (F) cells. *P<0.05, **P<0.01, ***P<0.001compared with the control group. BBR, berberine; CON, control

Figure 2. Growth inhibition of cisplatin in MCF-7 (A) and MDA-MB-231 (B) cells with and without berberine. Cisplatin cytotoxicity was significantly enhanced in when berberine was applied in combination. A combination treatment of doxorubicin with berberine did not show synergistic effect on MCF-7 (C) and MDA-MB-231 (D). *P<0.05, **P<0.01, ***P<0.001 compared with the same concentration groups treated with cisplatin or doxorubicin alone. PT, cisplatin; DOX, doxorubicin; BBR, berberine

Table 1. Percent Distribution of Cells in Different Phase of Cell Cycle by Flow Cytometry

Cell line	Treatment	Average % of cells in each phase		
		G0/G1	G2/M	S
MCF-7	Control	39.0	12.8	48.2
	20μM BBR	81.6	10.6	7.8
MDA-MB-231	Control	51.3	12.7	35.9
	20μM BBR	46.0	15.0	39.0

85 μM, respectively, the inhibitory effect was most potent in MDA-MB-231 cells. The cell viability was about 92% and 80% for the MCF-7 and MDA-MB-231 cells at 20 μM, respectively, as compared to their controls. For the subsequent experiments, we treated the breast cancer cells with the berberine at the 20 μM which no obvious cell death was detected in the culture.

To further test whether berberine suppresses growth of breast cancer cells, we performed colony formation assays in MCF-7 and MDA-MB-231 cells. Berberine markedly suppressed the colony formation abilities of the both breast cancer cells, compared with control groups (Figure 1). These data suggest that berberine suppresses cell proliferation of breast cancer.

Berberine enhances Cisplatin-mediated cytotoxicity in breast cancer cells

Subsequently, we investigated whether the combination of berberine and cisplatin or doxorubicin exhibited synergistic inhibitory effect on the growth of MCF-7 and MDA-MB-231 cell line. As shown in Figure 2, combination of cisplatin and berberine significantly increased the cytotoxicity in MCF-7 and MDA-MB-231 cell line with the IC$_{50}$ value of 15 and 18uM respectively, as compared with cisplatin alone with the IC$_{50}$ value of 21 and 23 uM respectively. However, a combination treatment with doxorubicin and berberine did not show a synergistic effect on both breast cancer cells.

Berberine induces G1-phase arrest in MCF-7 cell but not in MDA-MB-231 cell

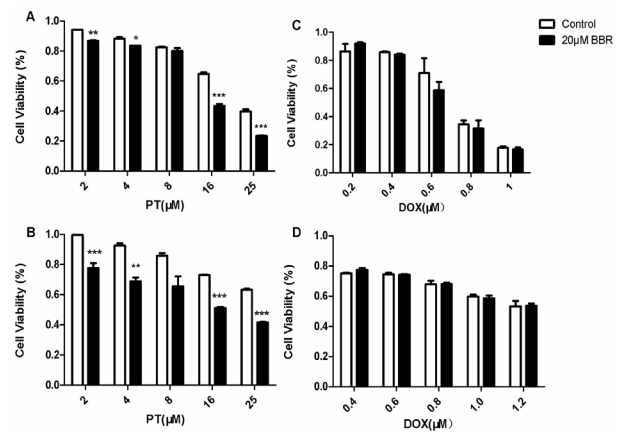The effect of berberine on cell cycle distribution was analyzed by using flow cytometry. As shown in Table 1, treatment of MCF-7 cells with berberine for 48h resulted in significantly higher percentage of cells at G1 phase (39.0 to 81.6%) and a concomitant lower percentage of cells at S phase (48.2 to 7.8%). However, no significant difference in cell cycle distribution was observed in MDA-MB-231 cells exposed to 20 μM berberine for 48h.

Berberine influences gene expression profiles assessed by microarray analysis

To identify the target genes regulated by berberine in breast cancers ER+ and ER- cells in more detail, we tested the effects of berberine on global gene expression profiles in MCF-7 and MDA-MB-231 cells by using Agilent human whole genomic 4×44K arrays. As compared with control groups, 1318 genes were up-regulated and 2079 genes were down-regulated more than two-fold by berberine in MCF-7 cells, while 1662 genes were up-regulated and 1044 genes were down-regulated more than two-fold in MDA-MB-231 cells. Among these genes, 451 genes regulated by berberine in both MCF-7 and MDA-MB-231 cells, 199 genes were found to be up-regulated and 142 genes down-regulated in these two cell lines. And 31 genes were found to be up-regulated in MCF-7 but down-regulated in MDA-MB-231, while 79 genes were found to be down-regulated in MCF-7 but up-regulated in MDA-MB-231 cells by berberine.

Database analysis of genes identified by microarray

To identify the precise gene function or pathway

Table 2. Gene Ontology Analysis of Genes Regulated by Berberine in Both MCF-7 and MDA-MA-231 Cells

| GO term	Count	*P*-value	Genes
Regulation of cell migration			
GO:0030334	10	9.8E-3	JAK2, APC, CXCR4, CORO1A, GNA13, ITGA2, LAMA3, PLD1, THBS1, B9D2
Response to drug			
GO:0042493	11	1.7E-2	ATG5, APC, CARD9, CYP1A1, GCLM, GNAO1, HDAC4, ITGA2, MAT2A, NOS3, B9D2
Apoptosis			
GO:0006915	21	3.1E-2	ATG5, E2F2, JAK2, CASP2, CXCR4, CDKN2A, CKAP2, DIDO1, DYRK2, EGLN3, GADD45A, HIPK2, LGALS7, RHOT1, RNF144B, SRGN, SGK1, THBS1, TNFRSF19, TNFAIP3, ZMAT3
Regulation of cell cycle			
GO:0051726	13	5.0E-2	ATRIP, CKS2, E2F2, H2AFX, MAD2L1, RAD17, APC, CENPE, CDKN2A, CYP1A1, GADD45A, PIM1, B9D2
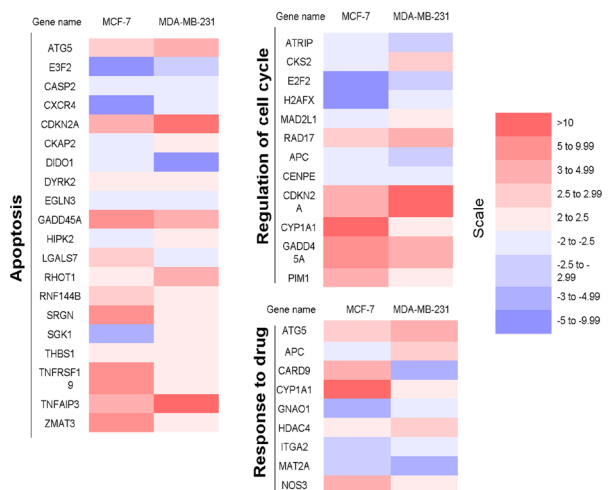
Figure 3. Assignment to Functional Groups of Genes Regulated by Berberine More than Two Fold in Both Breast Cancer Cell Lines. Color displays of the expression of those groups include apoptosis, cell cycle and response to drug

Figure 4. Verification of mRNA Expression of Selected Genes by qPCR Analysis. Fold change over vehicle from qPCR and microarray analysis is shown below the bars. *P<0.05, **P<0.01, ***P<0.001 compared with the control group. BBR, berberine

regulated by berberine, we used Gene Ontology (GO) assay to assess the function of the altered genes. The genes with expression altered by more than 2-fold were sorted into functional assay, genes related to regulation of cell cycle, cell migration, apoptosis, and drug response were identified (Table 2). Altered genes, which were regulated by berberine in both breast cancer, involved in the regulation cell cycle, apoptosis, and drug response were identified (Figure 3). Genes that were regulated by berberine more than 10-fold in MCF-7 and MDA-MB-231 cells.

Verification of microarray results using RT-PCR

To confirm the microarray data, qPCR analysis was conducted for ten selected genes. We selected candidate target genes based on previously reported association with breast cancer and GO analysis. Firstly; we verified seven target genes in MCF-7 cell regulated by berberine, as shown in Figure 4, four of these genes (TIMPP3, CDKN1A, SLC7A11, and E2F1) were altered in MCF-7 cell but not in MDA-MB-231 cell, and the others (GADD45A, CYP1A1, and CXCR4) were also regulated by beriberine in MDA-MB-231 cell. Notably, TIMP3, CDKN1A and SLC7A11 mRNA level were increased in berberine-treated MCF-7 cells as compared with the control cells, while E2F1 mRNA level was markedly decreased in the MCF-7 cells after treatment with berberine. Secondly, we investigated six target genes
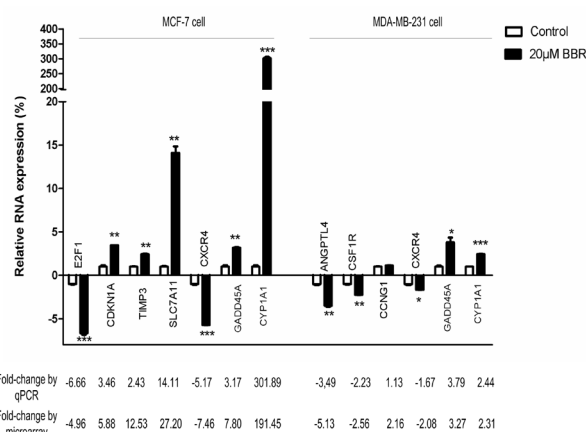
in MDA-MB-231 cell regulated by berberine, three of these genes (CCNG1, ANGPTL4, CSF1R) were altered in MDA-MB-231 cell but not in MCF-7 cell, and the other altered genes in both cell lines were same with the genes in MCF-7. The mRNA level of ANGPTL4 and CSF1R were significantly decreased in MDA-MB-231 cell after treatment with berberine as compared with control groups. Although CCNG1 was found to be up-regulated by berberine more than two folds by microarray assay in MDA-MB-231 cell, no statistically significant change in the level of CCNG1 mRNA was observed. At least, we compared three genes regulated by berberine in these two cell lines. CYP1A1 and GADD45A were significant up-regulation while CXCR4 was marked downregulation in both breast cancer MCF-7 and MDA-MB-231 cells. The qPCR and microarray expression results matched well, although there was variation in fold change for genes expression.

Discussion

Berberine is a naturally occurring isoquinoline alkaloid isolated from many kinds of medical herbs such as Copits chinensis, Coptis rhizome, Berberis aristata (Imanshahidi et al., 2008). It has been shown to exhibit a variety of pharmacological activities, including anti-diarrheas, anti-diabetes, anti-inflammatory, and anti-tumor capabilities (Kuo et al., 2005; Lee et al., 2006; Tillhon et al., 2012). In recent years, accumulating evidences have demonstrated

that berberine has inhibitory effects on different types of cancer cells such as colon cancer (Wu et al., 2011), prostate cancer (Meeran et al., 2008), liver cancer (Tan et al., 2006) and breast cancer (Kim et al., 2008a) by inducing cell cycle arrest, suppressing DNA synthesis, activating caspases and inducing apoptosis. Recently researches found that berberine induced cell cycle arrest in both breast cancer ER+ and negative ER- cells. In MDA-MB-231 cell, berberine inhibited cell invasion by suppressing AP-1 DNA binding activity (Kim et al., 2008b). In MCF-7 cells, berberine markedly inhibited cell proliferation and induced cell death through a mitochondria and caspase dependent apoptotic pathway (Patil et al., 2010). According to the previous reports, the same treatment may cause different results in different cells due to variation in the signaling pathways in response to berberine. Despite growing evidence has shown that berberine plays anti-tumor in a wide spectrum of cancers, very few studies has explored the different effects of berberine on breast cancers ER + and ER - cell lines together.

In this study, we demonstrated that berberine inhibits breast cancer ER + MCF-7 cell and ER - MDA-MB-231 cell growth. Moreover, a combination berberine with cisplatin resulted in synergistic inhibitory effect on both breast cancer cells. The inhibitory effect of berberine on MCF-7 might attributable to inducing G1 phase arrest, in accordance with previous reports (Kim et al., 2008a). To better understand the mechanisms of berberine action, we performed genome-wide expression profiling of berberine-treated cells using cDNA microarrays. To the best of our knowledge, this is the first report, which analyzed the gene expression profiles in response to berberine in both breast cancers ER + MCF-7 and ER - MDA-MB-231 cells together.

To verify the microarray data, qPCR analysis was conducted for ten selected genes based on previously reported association with breast cancer and GO analysis. Four genes regulated by berberine in MCF-7 cell but not in MDA-MB-231 cell were selected a detailed investigation of the mechanisms of growth inhibition of berberine on breast cancer ER + cell. Tissue inhibitors of MMP (TIMP3), which is up-regulated after berberine expose in MCF-7 cells, has been recognized as potential inhibitors of angiogenesis and cancer invasion and metastasis (Ahonen et al., 2002). Interestingly, it demonstrated that berberine suppressed the activities of MMP1, MMP2, MMP9 (Sekhon et al., 2008; Kim et al., 2012), therefore, our results suggested that berberine inhibited the activities of MMPs may be via up-regulation of TIMP3 expression. SLC7A11, which mediates cystine –glutamate exchange and thereby regulates intracellular glutathione (GSH) levels, play an important role in maintaining the higher GSH and consequently in cisplatin resistance (Okuno et al., 2003). Our study indicated that berberine induced SLC7A11 gene expression, further explained the mechanism of berberine enhancing cytotocity of cisplatin. We also indicated that berberine inhibited S-phase cell cycle progression of MCF-7 cell may be mediated, in part, by down-regulation of E2F1 (Chen et al., 2013). We also confirmed the data by Liu et al. that CDKN1A is regulated by berberine in MCF-7 cell (Liu et al.,

2009). Three genes regulated by berberine only in MDA-MB-231 cell were selected for a detailed investigation of the mechanisms of growth inhibition of berberine on breast cancer ER - cell. Recent studies have revealed an important role for ANGPTL4 in cancer proliferation, anoikis resistance, angiogenesis, and metastasis (Tan et al., 2012), and our finding suggested that berberine inhibited ANGPTL4 represents a novel molecular mechanism of its anticancer activity on MDA-MB-231 cells. CSF1R, which implicated in the pathogenesis and progression of breast cancer (Koren et al., 2000), was markedly decreased in berberine-treated MCA-MB-231 cells as compared with control. Although CCNG1 was found to be up-regulated by berberine more than two folds by microarray assay in MDA-MB-231 cell, no statistically significant change in the level of CCNG1 mRNA was observed. The reason of this difference maybe the use of different MDA-MB-231 clones. To further explore the common target genes in response to berberine in both MCF-7 and MDA-MB-231 cells, we investigated a set of altered genes at the same regulatory. CYP1A1, which is an important metabolism enzyme expressed in breast tissue, has been suggested to play a role in the metabolism of estrogens and the mammary carcinogens (Tsuchiya et al., 2005). CYP1A1 was significant up-regulation as compared with control groups in these two cells, especially in MCF-7 cells. Thus it can be assumed that berberine may shift estrogen metabolism, thereby decreasing the opportunity of genotoxic insult. The chemokine receptor CXCR4 has been suggested to be a prognostic marker in breast cancer; it plays a pivotal role in breast cancer especially in the metastatic spread of the disease (Balkwill F, 2004). Decreased expression of GADD45A has been reported in patients who suffer from breast cancer (Wang et al., 2005), although the precise mechanisms have not been clearly elucidated. Further functional studies are needed to assess the role of the array-identified genes in the berberine-mediated anti-cancer activities.

Regulation by berberine over a tenfold assumed that those targets can play a role in the anticancer effects of berberine. Further investigation should be required to clarify the molecular basis of berberine.

In conclusion, our study demonstrated an inhibitory role for berberine in both early stage of breast cancer ER + MCF-7 cells and advanced stage of breast cancer ER - MDA-MB-231 cells. Alteration of genes expression profiling may explain the inhibitory activity of berberine. However, further studies of gene functional analysis and in vivo trials need to be conducted.

Acknowledgements

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