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Basic Research Paper

Impact of epigallocatechin gallate on gene expression profiles of human hepatocellular carcinoma cell lines BEL7404/ADM and BEL7402/5-FU

Hai-Hua Tang, Ming Zhou and Gang Liang*

Department of Pharmacology; College of Pharmacy; Guangxi Medical University; Nanning, Guangxi P.R. China

Key words: cDNA microarray, epigallocatechin gallate, liver neoplasm, BEL7402 cell, multidrug resistance, reverse

Background and Objective: Epigallocatechin gallate (EGCG) from green tea could reverse multidrug resistance (MDR) in human hepatocellular carcinoma (HCC) in vitro and in vivo. This study was to investigate the mechanism of reversing effect of EGCG on MDR of human hepatocellular carcinoma cell lines BEL7404/ADM and BEL7402/5-FU. Methods: Drug sensitivity of BEL7404/ADM and BEL7402/5-FU cells was tested by MTT assay. The different gene expression profiles of BEL7404/ADM and BEL7402/5-FU cells were detected by cDNA microarray before and after treatment of EGCG. The expression of MDR1 and LRP genes was determined by reverse transcription-polymerase chain reaction (RT-PCR); the expression of Cyclin G1 protein was detected by Western blot to confirm the results of cDNA microarray. Results: The 10% inhibitory concentration (IC_{10}) of EGCG was 24.76 mg/L for BEL7404/ADM cells and 20.60 mg/L for BEL7402/5-FU cells. When treated with 0.05 mg/L adriamycin (ADM) and 100 μmol/L 5-fluorouracil (5-FU) in combination, 20 mg/L EGCG reversed the MDR by 9.66 folds in BEL7404/ADM cells and by 2.36 folds in BEL7402/5-FU cells. After treatment of EGCG, 210 differentially expressed genes were identified in BEL7404/ADM cells: 38 were upregulated and 172 were downregulated; the potential MDR-related genes included the upregulated ABCB10 (MDR/TAP), TOP2A, TOP2B, CCNG1, and downregulated ABCB1, MVP, ARHD, HDAC5, GSS, GSTPI, HSPA1B, HSPB7, CDKN1A, RAB11B, RAB9P40. After treatment of EGCG, 179 differentially expressed genes were identified in BEL7402/5-FU cells: 31 were upregulated and 148 were downregulated; the potential MDR-related genes included the upregulated ABCG (BCRP), CCNG2, GADD34, RB1, RBBP4, and downregulated DTYMK, GPX1, USP5, BAX, BAK1, HSPA1L. The downregulation of MDR1 and LRP expression was confirmed by RT-PCR; the upregulation of Cyclin G1 expression was confirmed by Western blot. Conclusion: EGCG could reverse the MDR of BEL7404/ADM and BEL7402/5-FU cells, but the changes of gene expression profiles of these two HCC cell lines are different.

*Correspondence to: Gang Liang; Department of Pharmacology; College of Pharmacy; Guangxi Medical University; Nanning, Guangxi, 530021 P.R. China; Tel.: 86.771.5358272; Fax: 86.771.5358272; Email: lianggang22@yahoo.com.cn

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Liver cancer is currently one of the malignancies with the poorest prognosis. Multi-drug resistance (MDR) is the biggest obstacle to its treatment. Looking for MDR reversal agent with high efficiency, low toxicity, and wide range of target sites from natural resources is the trend and hot-spot in the research of MDR. Epidemiologic results showed that green tea has antitumor effect on various tumors.[1] Tea leaf contains polyphenolic compounds that mostly are flavanols, generally known as catechin, in which epigallocatechin gallate (EGCG) is the most abundant and takes up 80% of the total. EGCG could inhibit the formation, growth and metastasis of tumor through many pathways, and has synergic effects with chemotherapeutic drugs on inhibiting proliferation and inducing differentiation and apoptosis in MDR cells of hepatic carcinoma, thus has the function of reversing MDR. In this study, gene chips was used to screen differentially expressed genes in different MDR hepatocellular carcinoma (HCC) cell lines after reversal with EGCG, to investigate possible target sites or mechanism for MDR reversal. This hopefully would provide an important theoretical evidence for the future research on structural modification of catechin as a tumor MDR reversal agent with high efficacy, expand the use of green tea in tumor treatment and boost up its therapeutic effect on HCC.

Materials and Methods

Materials. Cell lines. Human HCC cell lines BEL7404 and BEL7404/ADM were provided by the Fundamental Immunology Research Center of the Affiliated Tumor Hospital of Guangxi Medical University.[2] Human HCC cell lines BEL7402 and BEL7402/5-FU were provided by Nanjing Keygen Biotech Co., Ltd.

Primary reagents EGCG with a purity of 99.56%, was made by Zhejiang Yixin Pharmaceuticals. Cell culture reagents were from Gibco Company. Trizol reagent was manufactured by Shanghai Biotech Company. The cDNA chips were from Shenzhen Chipscreen Biotechnology Co., Ltd. SuperScript II RNase reverse transcriptase and dNTP were from Invitrogen. QIA nucleic acid extraction kit and Qiagene PCR purification kit were from Qiagen Company. CyScribe cDNA labeling kit was from Amersham Pharmacia Biotech. Rat anti-human Cyclin G1 monoclonal antibody (No. 11C8) was from

NeoMarkers. Horseradish peroxidase labeled goat anti-rat HRP antibody and the Western blot kit were from Canadian Immunochemical Pharmaceutical Company. DMSO was from Sigma-Aldrich.

Essential equipments Slide glasses for gene chips (superchips) were from US PE Company. Microarray equipment, scanner, and membrane-transferring apparatus for gene chips were from Amersham Pharmacia Biotech. Constant temperature hybridization incubator was from Shellab. Electrophoresis tank was from Bio-Rad. Gel imaging equipment was from Vilber. Ultraviolet-ray cross-linking equipment was from Viber Lourmat.

Drug sensitization test. BEL7404, BEL7404/ADM, BEL7402, and BEL7402/5-FU cells (2 x 10^5 to 5 x 10^5/mL) were seeded into 96-well plates (100 μL/well), respectively, and cultured overnight. Different concentrations of vincristine (VCR), cisplatin (DDP), adriamycin (ADM), and 5-fluorouracil (5-FU) were added to measure the MDR of the four cell lines. For each concentration, four replicate wells were set, with a final volume of 200 μL for each. Cells were incubated for 48 h. Afterward, 20 μL of MTT reagent (5 g/L) was added into each well and cells were cultured for another 4 h. After incubation, culture media was discarded, MTT crystals were dissolved with DMSO (150 μL/well) and sufficiently shaken for 10 min. The absorption value at 570 nm (A_{570}) was detected with a microplate reader setting at 570 and 630 nm to calculate cell inhibition rate according to the following formula: cell inhibition rate = (A value of negative control well - A value of drug well) / A value of negative control well x 100%. The 50% inhibition concentration (IC_{50}) was calculated by LOGIT software. Resistance index (IR) = (IC_{50} of drug-resistant cell line / IC_{50} of parental cell line). As described before, 75, 150, 285, 560 and 1120 mg/L of EGCG were added for measuring the cytotoxic effect of EGCG on HCC cells. Cells were treated with 14, 20, and 80 mg/L EGCG plus 0.2 mg/L ADM (for BEL7404 cells), 0.05 mg/L ADM (for BEL7404/ADM cells), 2.0 mg/L 5-FU (for BEL7402 cells), or 1.0 mg/L 5-FU (for BEL7402/5-FU cells), respectively, for 48 h to measure the sensitizating effects of EGCG. The reversal effects of non-cytotoxic concentrations (7, 15, 20 mg/L) of EGCG on sensitivity of HCC cells to ADM and 5-FU were measured, and the best reversal concentration of EGCG was selected for gene chip test. Reverse fold = IC_{50} for drug-resistant cell line / IC_{50} under the effect of chemotherapeutic drugs. The experiments were repeated at least thrice on different days.

Gene chip testing. **Gene chip preparation.** Human gene expression spectrum CSC-GE-80 gene chips, which contain 8 064 cDNA samples of target genes, were purchased from US Invitrogen Company. Another ten supplementary genes were selected from the IMAGE human cDNA database. They were cloned and purified by reverse transcription-polymerase chain reaction (RT-PCR), then dissolved in microarray buffer solution (3x SSC). The microarray of gene chips was automatically performed by GenIII array equipment. The glass slides were then hydrated for 2 h and dried at room temperature for 0.5 h. They were cross-linked by ultraviolet ray (400 mj), treated with 0.2% SDS, water, and 0.2 mol/L NaOH solution for 10 min, and dried.

Extraction and measurement of total RNA Cells were treated with non-cytotoxic concentration (20 mg/L) of EGCG plus 0.05 mg/L ADM (for BEL7404/ADM cells) or 100 μmol/L 5-FU (for BEL7402/5-FU cells). Untreated BEL7404/ADM and BEL7402/5-FU cells were used as control. Total RNAs were extracted from the cells using Trizol RNA extraction kit, isolated and purified by RNeasy Mini Kit, measured by agarose gel electrophoresis and ultraviolet ray spectrometer, and stored at -80°C.

Hybridization. The first strand of cDNA was labeled to prepare fluorescent probe. RNA was defrosted at room temperature, predegenerated at 70°C for 5 min, and then placed on ice for 5 min. The first strand cDNA synthesis reaction system was added into the PCR tube and handled according to the user manual for human gene expression spectrum gene chip. Fluorescent probe was purified by QIA quick PCR Purification Kit according to the manufacturer's instructions. The A_{260}, A_{550} and A_{650} of the probe were measured using enzyme-labeled plate. The labeled probe (Cy3/Cy5) hybridization incubator was placed on a shaker in dark. Afterward, gene chips were washed subsequently with 0.2x SSC/0.2% SDS and 0.2x SSC at 50°C for 10 min for three times, dried, then scanned or placed in chip box in dark.

Gene chip scanning and data analysis Gene chips were scanned by Generation III Array Scanner and analyzed by ImageQuant 5.0 software. the images were overlapped by two kinds of fluorescence: signal points with strong Cy3 (in green) represented downregulation; while signal points with strong Cy5 signal (in red) represented upregulation; signal points with signals of equally strong (in yellow) represented non-variation in expression. The images were split into single fluorescence images by Split-tiff software, loaded into image analyzing software, called ArrayVision, to be converted into numerical values. Then, they were loaded into analyzing software called MIDAS. The Cy5/Cy3 hybridization signal ratios were normalized by LOWESS method. A ratio of > 2 or < 0.5 indicated effective gene expression variation.

Expression of MDR1 and LRP detected by RT-PCR. The synthesis of cDNA cDNA was synthesized with 1 μg of total RNA using 1 μL reverse transcriptase M-MLV (20 U/μL) in a 20 μL reaction system, which was established under the existence of Random Primer, according to the instructions of the test kit.

PCR reaction The sequences were 3'-CCC ATC ATT GCA ATA GCAGG-5' for the upstream primer and 3'-GTT CAA ACT TCT GCT CCTGA-5' for the downstream primer of MDR1, 3'-TTC TGG ATT TGG TGG ACGC-5' for the upstream primer and 3'-ACT TCT CTC CCT TGA CCAC-5' for the downstream primer of LRP, 3'-ACC CCC ACT GAA AAA GATGA-5' for the upstream primer and 3'-ATC TTC AAA CCT CCA TGATG-5' for the downstream primer of β_2-MG. A reaction system of 50 μL was established with 5 μL 10xPCR buffer, 1 μL of each dNTP (10 mmol/L), 2.4 μL of $MgCl_2$ (25 mmol/L), 1 μL of downstream primer and 1 μL of upstream primer of MDR1, LRP and β_2-MG. After pre-degeneration at 94°C for 10 min, the reaction system was added with 1.2 U Taq DNA polymerase, then followed by 30 cycles of degeneration at 94°C for 40 s, annealing at 52°C for 40 s, and extension at 72°C for 50 s. PCR products of MDR1 (157 bp), LRP (285 bp) and β_2-MG (189 bp) were analyzed with 2.5% agarose gel electrophoresis.

Expression of Cyclin G1 protein detected by Western blot. Cells were collected and washed three times with ice-cold phosphate-buffered saline (PBS), then lysed on ice with adequate protein lysis buffer for 30 min. Cell lysates were centrifuged at 10,000x g at 4°C for 20 min, and the supernate was collected. The concentration of proteins were measured by Bradford method. The samples were

Table 1 **The Cytotoxic and drug-sensitization effects of EGCG on four hepatocellular carcinoma cell lines**

Drug (mg/L)	Survival rate (%)			
	BEL7404	BEL7404/ADM	BEL7402	BEL7402/5-FU
EGCG 75	94.2±9.7	94.0± 8.6	91.3±7.1	92.5±4.2
150	83.3±7.7	92.1±11.2	77.9±6.2	73.4±3.9
285	55.4±6.5	85.2± 7.6	60.1±4.3	58.8±2.4
560	47.1±5.4	73.8± 9.1	52.8±8.1	52.1±5.7
1120	37.6±7.4	69.9±10.3	38.8±9.4	49.7±4.4
ADM(0.2)	52.3±4.9	78.6± 8.4	–	–
5-FU(2.0)	–	–	51.6±3.8	85.0±7.9
EGCG (14) + ADM (0.2/0.05)	42.3±5.7[a]	29.4± 3.8[a]	–	–
EGCG (20) + ADM (0.2/0.05)	38.8±5.1[a]	20.7± 6.8[a]	–	–
EGCG (80) + ADM (0.2/0.05)	33.6±6.9[a]	20.2± 5.5[a]	–	–
EGCG (14) + 5-FU (2.0/1.0)	–	–	54.2±9.7[a]	31.3±4.8[a]
EGCG (20) + 5-FU (2.0/1.0)	–	–	49.4±2.7[a]	25.2±1.7[a]
EGCG (80) + 5-FU (2.0/1.0)	–	–	34.5±3.3[a]	24.9±4.2[a]

EGCG, epigallocatechin gallate; ADM, adriamycin; 5-FU, 5-flurouracil. All values are presented as mean ± SD of nine experiments. [a]$p < 0.01$, vs. ADM or 5-FU.

Table 2 **The reversal effect of EGCG on resistance of BEL7404/ADM cells to ADM**

Group	IC_{50} of ADM (mg/L)	RF
Control	3.85±0.16	–
VRP 5 mg/L	0.59±0.12	6.48[a]
EGCG 7 mg/L	1.42±0.34	2.69
15 mg/L	0.72±0.18	5.37[a]
20 mg/L	0.39±0.14	9.66[ab]

VRP, verapamil. All values are presented as mean ± SD of three experiments. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. VRP group.

Table 3 **The reversal effect of EGCG on resistance of BEL7402/5-FU cells to 5-FU**

Group	IC_{50} of 5-FU (μmol/L)	RF
Control	2 833.62±201.03	–
VRP 5 mg/L	2 257.43±110.34	1.24
EGCG 7 mg/L	2 096.78±145.21	1.35
15 mg/L	1 816.31±103.32	1.56[a]
20 mg/L	1 197.27± 89.71	2.36[ab]

All values are presented as mean ± SD of three experiments. [a]$p < 0.05$, vs. control group; [b]$p < 0.05$, vs. VRP group.

loaded for electrophoresis in 10% separation gel and 5% SDS-PAGE, with half-dried method and at constant current of 100 mA. They were transferred to membrane for 1.5 h, and blocked by 3% BSA at 4°C overnight. After immunological reaction, they were colorized by horseradish peroxidase (HRP-ECL) and scanned.

Statistical analysis. Data were presented as mean ± SD. Multiple comparison was performed by analysis of variance (ANOVA) using SPSS 13.0 software.

Results

Drug resistance of hepatocellular carcinoma cells. The IC_{50} of ADM were 0.13 mg/L for BEL7404 cells and 3.85 mg/L for BEL7404/ADM cells. The drug resistance index of BEL7404/ADM cells was 39.6. BEL7404/ADM cells showed cross-resistance to DDP and 5-FU, but not to VCR. The IC_{50} of 5-FU were 22.21 μmol/L for BEL7402 cells and 2833.62 μmol/L for BEL7402/5-FU cells. The drug resistance index of BEL7402/5-FU cells was 127.6. BEL7402/5-FU cells showed cross-resistance to ADM, but not to DDP and VCR.

Cytotoxicity and reversal effect of EGCG on drug resistance. No apparent cytotoxicity was seen in EGCG at concentrations under 100 mg/L. As the concentration of EGCG increased from 100 mg/L, the cell survival rates of all four HCC cell lines decreased gradually as well; the cell inhibition rate was significantly lower in single EGCG group than in combination group (Table 1). Non-cytotoxic concentrations (7, 15, 20 mg/L, lower than IC_{10}) of EGCG significantly reversed the resistance of BEL7404/ADM cells to ADM; the reversal effect of 20 mg/L EGCG was significantly stronger than 5 mg/L VRP (Table 2). Non-cytotoxic concentrations (15 and 20 mg/L) of EGCG significantly reversed the resistance of BEL7402/5-FU cells to 5-FU; the reversal effect of EGCG was significantly stronger than that of VRP (Table 3).

The testing results of gene chips. After hybridization of gene chips, the fluorescent signal intensity was high, with even background and without obvious blank spot (Fig. 1). As seen in the scatter plot of Cy3/Cy5 signal correlation of the gene chip (Fig. 2), when treated with EGCG at non-cytotoxic concentrations, the gene expression profile of BEL7404/ADM cells was similar to that of BEL7402/5-FU cells, suggesting that EGCG, under the same concentrations, had same effects on both cell lines. However, a few differentially expressed genes were also noticed, suggesting that EGCG functioned differently on these two cell lines under different mechanisms.

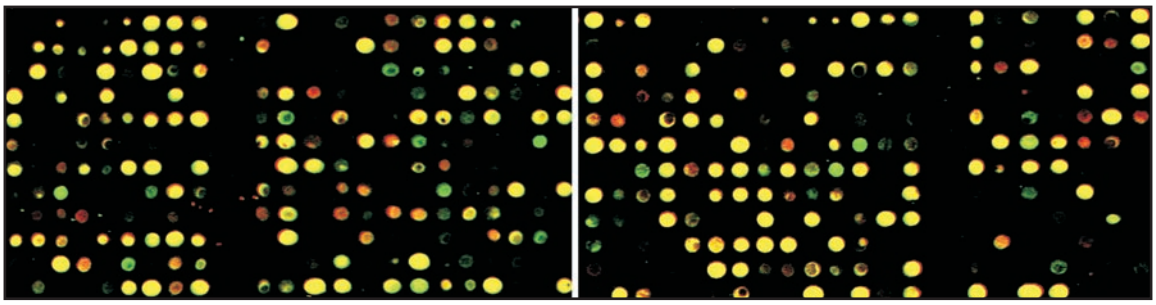

Figure 1. Gene expression profiles of BEL7404/ADM and BEL7402/5FU cells after treatment of EGCG detected by cDNA microarray

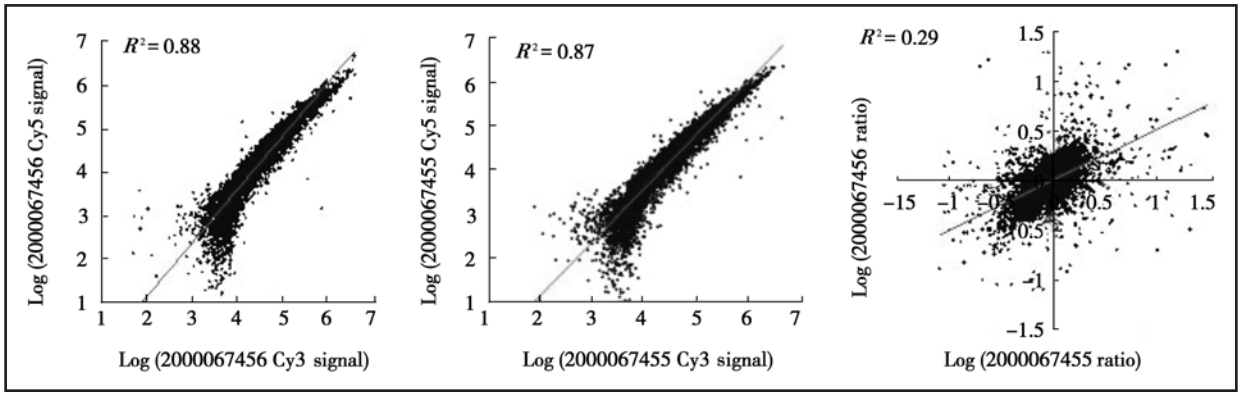

Figure 2. Scatter plot of Cy3/Cy5 hybridization signal of BEL7404/ADM and BEL7402/5-FU cells after treatment of EGCG detected by cDNA microarray.

In BEL7404/ADM cells, 210 genes were differentially expressed in the experiment of dual fluorescent pathways, including 38 upregulated and 172 downregulated; 50 genes were differentially expressed in the experiment of single fluorescent pathway, including 15 upregulated and 35 downregulated (Table 4). In BEL7402/5-FU cells, 179 genes were differentially expressed in the experiment of dual fluorescent pathways, including 31 upregulated and 148 downregulated; 66 genes were differentially expressed in the experiment of single fluorescent pathway, including 13 upregulated and 53 downregulated (Table 5). Primary analysis revealed that these differentially expressed genes were mainly classified into three groups: cellular proliferation & apoptosis regulation genes, tumor-related genes, and gene transcription-related genes.

Expression variations of MDR1 and LRP in BEL7404/ADM and BEL7402/5-FU cells after treatment of EGCG. As shown in Figure 3, when BEL7404/ADM cells were treated with EGCG plus ADM and BEL7402/5-FU cells were treated with EGCG plus 5-FU for 48 h, the mRNA levels of MDR1 were reduced by 45.3% and 20.9% of control, respectively, while the mRNA levels of LRP were reduced by 43.4% and 15.2% of control, respectively. These results were consistent with the gene chip results, proving that the gene chip testing results were reliable.

Expression variations of Cyclin G1 protein in BEL7404/ADM and BEL7402/5-FU cells after treatment of EGCG. As shown in Figure 4, when BEL7404/ADM cells were treated with EGCG plus ADM and BEL7402/5-FU cells were treated with EGCG plus 5-FU for 48 h, the protein levels of Cyclin G1 were increased by 2.86 and 0.95 folds of control, respectively. The result was in accordance to the expression variation of CCNG1 gene in the cDNA microarray, proving that the gene chip testing results were reliable.

Discussion

In this study, the variations of gene expression profiles in two HCC MDR cell lines after treatment of EGCG were not completely the same. In BEL7404/ADM cells, the expression of human MVP gene, which encodes lung drug-resistant protein (LRP), and ABCB10 (MDR/TAP) showed significant changes, indicating that it could be helpful in improving the intercellular distribution of EGCG and the effect of chemotherapeutic drugs after entering nucleus. This is consistent with the inhibition of LRP gene expression in KBV200 transplanted tumor by EGCG.[3] The ratio of ABCB1 (MDR1) gene was less than 1, but its expression was not significantly decreased. The significant downregulation of HDAC5 gene suggests that the downregulation of MDR1 by EGCG is possibly related to the inhibition of deacetylase. The upregulation of DDX3 gene and the significant downregulation of DDX9 gene of single fluorescent pathway might be factors for influencing the expression of MDR1. The expression of TOP2A and TOP2B genes were increased by 1.62 and 1.63-fold. Even though these differences lack significance, to some degree, EGCG increased the amount of TOPO enzymes, which is the same as in the in vivo experiment.[4] The expression of GSS and GSTPI genes were downregulated, which influenced the activity of GSH. The downregulation of HSPA1B and HSPA1A genes, as well as the gene encoding heat-shock protein 70 (HSP70), are in line with the results reported by Ling et al.[5] The expression of another molecular chaperon gene CCT1 was downregulated, suggesting that

Table 4 **Differentially expressed genes in BEL7404/ADM cells after treatment of EGCG**

GenBank access No.	Gene function	Gene tag	Ratio
Up-regulated			
R83876	ATP-binding cassette, sub-family B (MDR/TAP), member 10	ABCB10	2.20
AA626845	DEAD/H(Asp-Glu-Ala-Asp/His)box polypeptide 3	DDX3	2.14
AA082943	Cyclin G1	CCNG1	2.22
H18068	Protein kinase C-like 1	PRKCL	10.42
AA019459	Protein tyrosine kinase 9	PTK9	2.35
H17504	Mitogen-activated protein kinase 6	MAPK6	2.07
R53787	Protein phosphatase 2, regulatory subunit B (B56), epsilon isoform	PPP2R5E	2.23
Down-regulated			
AA158991	Major vault protein	MVP	0.19
AA463458	Glutathione synthetase	GSS	0.46
AI963715	Heat shock 70 ku protein 1B	HSPA1B	0.38
AA045503	Heat shock 27 ku protein family, member 7 (cardiovascular)	HSPB7	0.40
AA676588	Chaperonin containing TCP1, subunit 7 (eta)	CCT7	0.49
AA454813	Interferon-related developmental regulator 2	IFRD2	0.42
AA443300	Matrix metalloproteinase 15 (membrane-inserted)	MMP15	0.33
AA465536	Ubiquitin specific protease 5 (isopeptidase T)	USP5	0.32
N64628	Ubiquitin-like 4	UBL4	0.40
AA496810	Protein kinase C substrate 80K-H	PRKCSH	0.38
AA256597	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	NFKB2	0.45
AI560680	Protein kinase C, alpha binding protein	PRKCABP	0.32
AA458993	Protein kinase C, zeta	PRKCZ	0.47
H18068	Protein kinase C-like 1	PRKCL1	0.42
R80779	Mitogen-activated protein kinase kinase kinase 11	MAP3K11	0.43
AA425826	Mitogen-activated protein kinase kinase 2	MAP2K2	0.49
AA143437	Ras homolog gene family, member	ARHD	0.39
AA626178	RAB5C, member RAS oncogene family	RAB5C	0.48
H84815	Rab9 effector p40	RAB9P40	0.37
R51209	Protein phosphatase 2A, regulatory subunit B' (PR 53)	PPP2R4	0.35
AI921113	Histone deacetylase 5	HDAC5	0.40
Reference			
AA424804	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	ABCC1	1.15
AA504348	Topoisomerase (DNA) Ⅱ alpha (170 ku)	TOP2A	1.62
AA489647	Cyclin G2	CCNG2	1.95
T59934	Topoisomerase (DNA) Ⅱ beta (180 ku)	TOP2B	1.63
W63749	B-cell CLL/lymphoma 2	BCL2	1.45
AA455911	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	0.87
AA487429	ATP-binding cassette, sub-family B (MDR/TAP), member 2	ABCB2	0.97
AW072826	BCL2-associated X protein	BAX	0.57
AA931820	BCL2-like 1	BCL2L1	0.94
AA291323	BCL2-interacting killer (apoptosis-inducing)	BIK	0.67
AI952615	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	0.53

Table 5 **Differentially expressed genes in BEL7402/5-FU cells after treatment of EGCG**

GenBank access No.	Gene function	Gene tag	Ratio
Up-regulated			
H52673	BCL2/adenovirus E1B 19 kD-interacting protein 3-like	BNIP3L	2.08
H20743	Growth arrest and DNA-damage-inducible 34	GADD34	2.45
AA082943	Cyclin G1	CCNG1	2.00
AW072826	Cyclin G2	CCNG2	3.42
R27585	Protein tyrosine phosphatase, receptor type, B	PTPRB	2.04
AI954093	Retinoblastoma-binding protein 4	RBBP4	3.05
Down-regulated			
AA428365	Ubiquitin specific protease 5 (isopeptidase T)	USP5	0.35
AA464256	Deoxythymidylate kinase (thymidylate kinase)	DTYMK	0.44
AI565972	Heat shock 70 ku protein-like 1	HSPA1L	0.47
R37276	Deoxythymidylate kinase (thymidylate kinase)	DTYMK	0.45
N20338	Interferon-related developmental regulator 2	IFRD2	0.41
AA454813	Insulin-like growth factor binding protein 2 (36 ku)	IGFBP2	0.38
T62547	Jun B proto-oncogene	JUNB	0.48
H79047	Ras-GTPase activating protein SH3 domain-binding protein 2	KIAA0660	0.48
N94468	Malignant cell expression-enhanced gene/tumor progression-enhanced gene	LENG4	0.40
AA281945	MKP-1 like protein tyrosine phosphatase	MKP-L	0.35
AA449667	Matrix metalloproteinase 15 (membrane-inserted)	MMP15	0.30
T54298	Protein phosphatase 2A, regulatory subunit B′ (PR 53)	PPP2R4	0.41
AI560680	Protein kinase C substrate 80K-H	PRKCSH	0.40
AA496810	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13	PSMD13	0.48
AI356337	BCL2-associated X protein	BAX	0.45
AA446477	Glutathione peroxidase 1	GPX1	0.47
AA465536	Eukaryotic translation initiation factor 4 gamma, 1	EIF4G1	0.14
AA017383	Eukaryotic translation initiation factor 5A	EIF5A	0.33
Reference			
AA455911	ATP-binding cassette, sub-family B (MDR/TAP), member 1	ABCB1	1.29
R83876	ATP-binding cassette, sub-family B (MDR/TAP), member 10	ABCB10	1.34
W84773	ATP-binding cassette, sub-family G (WHITE), member 2	ABCG2	1.79

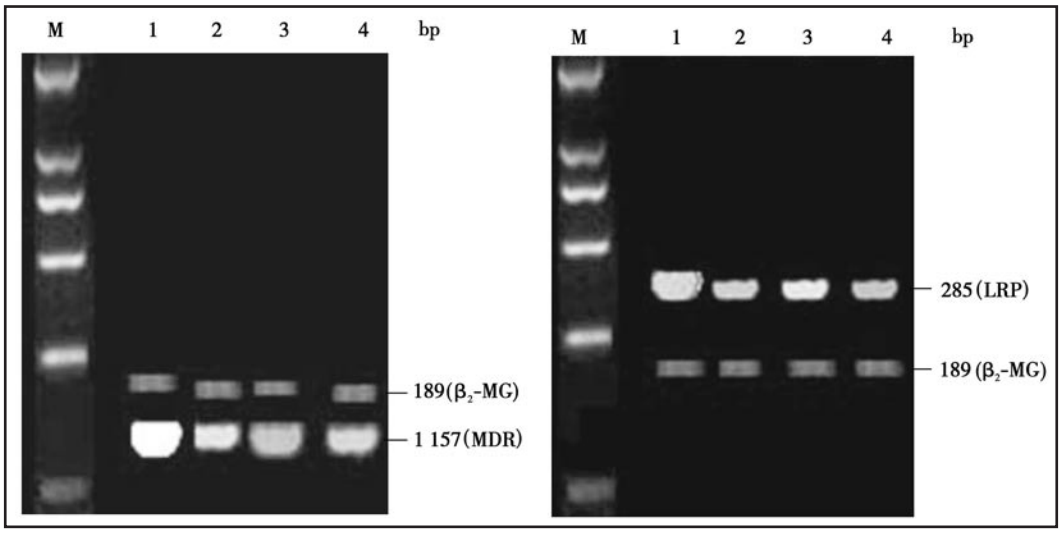

Figure 3. Expression of MDR1 and LRP in BEL7404/ADM and BEL7402/5-FU cells detected by RT-PCR. Lane M: marker; lane 1: untreated BEL7404/ADM cells; lane 2: BEL7404/ADM cells treated with 20 mg/L EGCG and 0.05 mg/L ADM for 48 h; lane 3: untreated BEL7402/5-FU cells; lane 4: BEL7402/5-FU cells treated with 20 mg/L EGCG and 100 μmol/L 5-FU for 48 h.

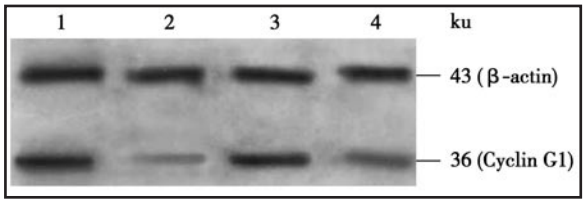

Figure 4. Effects of EGCG on Cyclin G1 expression in BEL7404/ADM and BEL7402/5-FU cells. Lane 1: BEL7404/ADM cells treated with 20 mg/L EGCG and 0.05 mg/L ADM for 48 h; lane 2: untreated BEL7404/ADM cells; lane 3: BEL7402/5-FU cells treated with 20 mg/L EGCG and 100 μmol/L 5-FU for 48 h; lane 4: untreated BEL7402/5-FU cells.

EGCG could reduce the protection of tumor MDR cells by HSP70, avoid apoptosis, and promote aging of cells, or downregulate the expression of HSP70 gene under stressful condition, activate the stress kinase and apoptotic gene, and promote apoptosis. The downregulation of genes encoding PKC and protein phosphatase may due to the inhibition of EGCG phosphorylation. However, no obvious change in apoptosis-related genes was seen in BEL7404/ADM cells, suggesting that EGCG of non-cytotoxic concentrations has no direct apoptotic effect on MDR cells induced by ADM.

Yuan et al.[6] first discovered that BCRP could bind 5-FU and pump out it from cells, therefore, specifically mediate the tolerance to 5-FU. Although the upregulation of ABCG (BCRP) in BEL7402/5-FU cells in this study was not significant, its ratio had reached 1.79, which might relate to the induction of tolerance in HCC by 5-FU. No significant change in other ABC transmembranous proteins was seen in BEL7402/5-FU cells, which may relate to drug resistance mechanism other than relying on ATPase to pump out drugs. Also, downregulation of GPX1 gene indicates that glutathione enzyme system might participate in MDR reversal of tumors. In the aspect of apoptosis regulation, DTYMK gene that encodes thymidine kinase (TK) could induce thymine-deficient death, which result in apoptosis and reduce the therapeutic effect of 5-FU.[7] The upregulation of GADD34 gene, which contains a P53-binding site and a negative regulatory factor for G_1/S phase check point,[8] could promote cell apoptosis. In addition, the expression of many apoptosis-inhibitory genes, such as HSPA1L gene of HSP family, GPX1 and IGFBP2 (insulin-like growth factor 2 binding protein), were downregulated, suggesting that EGCG could restore the sensitivity of BEL7402/5-FU cells to chemotherapy and promote apoptosis via regulating the expression of apoptosis-related genes. The gene for ubiquitin specific proteasome (USP 5C), ubiquitin-related gene (E2-EPF) and PSMD13 (subunit 13 of 26S proteasome) were downregulated, which could mediate protein hydrolysis, regulate cell cycle and signal transduction through ubiquitin pathway. Upregulation of RB1 gene, which regulates G_1 to S phase progression of cell cycle, and the coding gene for RBBP4 protein, as well as CCNG1 and CCNG2, which both regulate G_2/M phase, suggests that EGCG promotes the apoptosis of BEL7402/5-FU cells mainly through regulating cell cycle.

MMP15 expression was significantly decreased in both BEL7404/ADM and BEL7402/5-FU cells. Whether this is due to the inhibition of MDR cell growth by EGCG requires further investigation. No change of bcl-2 expression was observed in both cell lines, indicating that EGCG promotes cell apoptosis via enhancing the cytotoxicity of chemotherapeutic drugs, in stead of induce apoptosis directly. Other apoptosis-regulatory genes and related genes (such as BNIP3L and GADD34) also showed various degrees of changes. To elucidate whether the expression changes of apoptosis-related genes and EGCG of non-cytotoxic concentrations participate in MDR reversal of both cell lines as well as their relationship, the genes participate in cell cycle regulation and in signal transduction pathways (such as tyrosine kinase pathway, Ras-MAPK pathway, and NFκB activation pathway) should take into consideration. We previously found that EGCG could reverse the MDR of tumor cells through its synergetic effect with VCR on arresting tumor cells at G_2/M phase, which may via strengthening checkpoints of cell cycle.[4,9-11] The expression of CCNG1 and CCNG2, which encode Cyclin G, were upregulated in both cell lines. Cyclin G might mainly participate in cell cycle regulation and DNA repair. Therefore, in this study, Cyclin G1 was selected for protein verification by Western blot. However, the upregulation of CCNG1 and CCNG2 expression was not synchronous in both cell lines, which may due to their different regulatory mechanisms in cell cycle. Also, gene expression changes in multiple signal transduction pathways were synchronously observed. Many genes, such as PTK9, PTPRG, RAB26, and MAPK6, in the Ras-MAPK signal pathway (one of the downstream signal pathways of tyrosine kinase receptor) were upregulated, while RAB5C, RAB9P40, PTPN14, and MAP2K2 were downregulated. Asynchronous changes in genes for Ras-MAPK signal transduction suggest that they might inhibit DNA transcription and induce apoptosis. PITPNM and PRKCL1, encoding phosphatidylinositol transfer proteins, were downregulated, suggesting that NFκB pathway is involved in EGCG-induced apoptosis in MDR cells. PRKCL1, encoding protein kinase C (PKC), was downregulated, while PIASY, the STAT inhibitory protein, was downregulated, suggesting that their complementary inhibitory effects can not mediate cell apoptosis directly.

In summary, the gene expression alterations caused by EGCG in HCC MDR cells involve multiple genes, multiple pathways, and multiple target sites, which can be classified as transmembranous transport proteins and drug resistance-related enzymes and proteins, and relate to apoptosis-related genes, cell cycle regulation, and signal transduction pathways such as Ras-MAPK.

Gene chip, as an important technical platform for analysis system with high throughput, is based on molecular or cellular models. When combines with molecular hybridization principle, biotechnology, and computer analysis, it can be used to efficiently analyze a large amount of biological samples with high throughput and high sensitivity.[12] It could only provide initial information for thoroughly exploring the mechanism of drug action, which could not fully present the functions of their products. Also, the MDR mechanisms involve multiple pathways. DNA microarray is influenced by gene expression and regulation delay to some extent. Thus, it is difficult to explain simultaneous changes of these genes. However, to explore the complex mechanisms of tumor MDR and to research multiple targets for MDR reversal, gene chip has an advantage of high throughput over other single-factor analysis approach.

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