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ORIGINAL ARTICLE

Oligo-microarray analysis reveals the role of cyclophilin A in drug resistance

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Abstract Cyclophilin A (CYPA) belongs to peptidyl prolyl isomerases (PPIases), which catalyze the *cis*/*trans* isomerization of prolyl peptide bonds in cellular communication. CYPA has been implicated in several pathological processes, including cancer, inflammatory diseases, and HIV-1 infection. Up-regulation of *CYPA* has been found to be a common phenomenon in several tumor types, including in hepatocellular carcinoma (HCC). However, the role of CYPA in tumor cells remains unknown. We generated a stable SK-Hep1 cell line and studied the CYPA regulated genes at the transcriptome level. The microarray results reveal that CYPA can up-regulate the expression of many cytokine and drug resistance related genes. Furthermore, we showed that the elevated CYPA expression contributes to drug resistance. We postulate that the over-expression of CYPA in tumors may play a role in clinical resistance to chemotherapy.

Keywords Oligo-microarray · Peptidyl–prolyl isomerase (PPIase) · Cyclophilin A (CYPA/PPIA) · Hepatocellular carcinoma (HCC) · Multi-drug resistant protien (MRP)

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Introduction

Peptidyl prolyl isomerases (PPIases) are enzymes that catalyze the *cis*/*trans* isomerization of prolyl peptide bonds [1]. The PPIase superfamily is comprised of three distinct classes that differ in terms of protein structure and substrate specificity: cyclophilins (CYPs), FK506 binding proteins (FKBPs), and parvulins [2]. All members of the PPIase superfamily have been shown to accelerate protein folding reactions by catalyzing *cis*/*trans* isomerization of prolyl bond, and this step is a rate limiting process in protein folding [2]. Cyclophilins and FKBPs were also called 'immunophilins', for they are the cellular targets for the immunosuppressive drugs cyclosporin A (CsA) and FK506, respectively [3–5]. The CYPA/CsA and FKBP12/FK506 complexes each inhibit calcineurin, which is a key protein in T-cell activation. The isomerase activity of CYPA and FKBP12 is not directly involved in calcineurin inhibition directly [6].

As a prototypical member of the cyclophilin protein family, CYPA has been implicated in several pathological processes, including cancer [7, 8], rheumatoid arthritis (RA) [9, 10], and HIV-1 infection [11–13]. Up-regulation of *CYPA* has been found to be a common phenomenon in several tumor types [14], and knockdown expression of CYPA by RNAi diminishes non-small-cell lung tumor growth in vivo [15]. In rheumatoid arthritis patients, over-expressed CYPA stimulates the production of inflammatory cytokines, such as TNF-a, interleukin-1β (IL-1β), IL-8, monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase 9 (MMP-9) [10]. During the process of HIV-1 infection and replication, CYPA can be incorporated into HIV-1 virions, and facilitates the processing of capsid core disassembly [11, 12, 16].

Previous proteomic analysis showed that CYPA is over-expressed in hepatocellular carcinoma (HCC) [17], while the roles of CYPA in tumor cells remain unknown. We stably expressed human CYPA in hepatocyte SK-Hep1 and studied the deregulated genes at the transcriptome level. The microarray results revealed that CYPA can up-regulate the expression of many cytokine related genes, drug transport- and drug metabolism-related genes. Further experiments illustrated that CYPA over-expression contributes to drug resistance, and may play roles in clinical resistance to chemotherapy.

Materials and methods

Tissue samples

Paraffin-embedded sepecimens of 46 cases of HCCs were obtained from tissue bank of Institute of Liver Cancer Research of Zhongshan Hosiptal and Institute of liver cancer of Qidong of Jiangsu Province. Tissue samples were reviewed by a pathologist from corresponding hospital. Paraffin-embedded sepecimens of 12 nonmalignant samples, which include ten cases of hepatocirrhosis and two normal cases, were from The First People Hospital Shanghai attached to Jiaotong University and department of Histology and Embryology of Shanghai Medical College, respectively.

Secondary biotinlyated antibody goat-anti-rabbit and SABC complex and DAB (3,3′-diaminobenzidine tetra hydrochloride) staining kit were purchased from Boster (Wuhan, China).

Immunohistochemistry

Each section was sliced into 4 μm sections. The sections were deparaffined in xylene and rehydrated in alcohol, endogenous peroxidase was perished by 3% H_2O_2 for 10 min. Antigen retrieval was achieved by treatment of microwave in citrate acid buffer (pH 6.0) for two times, and then blocked in 3% goat serum (Boster China) for 30 min in 37°C for any none specific reaction, and incubated in primary antibody in 4°C overnight, washed in PBS for three times, incubated in biotinlyated secondary antibody (Boster China) for 30 min, washed in PBS (0.01, pH 7.2) for three times, incubated in SABC complex (Boster China) for 25 min, washed in PBS (0.01, pH 7.2) for three times, immunostaining was visualized by using 3,3′-diaminobenzidine tetra hydrochloride (DAB/H_2O_2; Boster China) and counterstained with hemtoxylin. immunohistochemistry of each tissue was repeated three times. The tissues were considered positive only when distinct cytoplasm staining was identified.

Plasmids

The coding sequences of human CYPA and CYPJ were inserted in-frame in to plasmids pCMV-HA (Novagen) and pcDNA3.1-myc (Novagen). Then we got the plasmids pCMV-CYPA/CYPJ for transient transfection, and plasmid pcDNA3.1-CYPA/CYPJ for stable transfection.

Cell culture and stable transfection

The SK-Hep1 cells (American Type Culture Collection, ATCC) were cultured in RPMI-1640 medium (Gibco-BRL, Grand Island, NY, USA) that contained 10% fetal bovine serum (FCS; Gibco-BRL). Cells were cultured in 37°C with 5% CO_2. The pcDNA3.1-CYPA or pcDNA3.1-CYPJ plasmid was transfected into the SK-Hep1 cells (hepatocellular carcinoma) using LipofectAMINE 2000 reagent (Invitrogen) under manufacturer's protocol. After 2 days, the cells were transferred to media containing 600 μg/ml G418 (Life Technologies Inc.). The surviving cells were grown under continuous selection with G418. Three weeks later, the surviving cells were analyzed by Western blotting and quantitative real-time PCR analyses to detect the expression of CYPA and/or CYPJ. SK-Hep1 cells were also transfected with empty vector (pcDNA3.1-myc) in a similar fashion and this stable cell line was used as control.

Antibodies

Anti-myc and anti-HA monoclonal antibodies and anti-β-actin rabbit polyclonal antibody were purchased from Cell Signaling Technology (Danvers, MA; catalog number 2367, 2276, and 4967). Rabbit anti-CYPA polyclonal antibody was obtained from Ambion Company (Austin, TX; catalog number AM4309). Western blot was performed under methods that have been described previously [18].

Oligo-microarray analyses

Total RNA of SK-Hep1-CYPA and SK-Hep1-pcDNA was prepared using Trizol reagent (Invitrogen, Carlsbad, CA, USA), purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and RNA quality was judged by denaturing agarose gel electrophoresis. Hybridizations were performed at Gene Company Ltd. (Shanghai). Double-stranded DNA was synthesized using one-cycle cDNA Synthesis Kit followed by purification with GeneChip Sample Cleanup Module (Affymetrix). The double-stranded DNA was used as template for the in vitro transcription using GeneChip IVT Labeling Kit yielding biotin-labeled cRNA. Following cleanup and quantifying spectrophotometrically, the purified cRNA was fragmented into short sequences. Eighty microliters of the hybridization cocktail was first

hybridized to the test-chips to check the cRNA integrity and the system veracity. Then the HG-U133 plus 2.0 microarrays with 39,000 transcripts (Affymetrix) were directly loaded with 200 µl of hybridization cocktail solution and placed in Genechip Hybridization Oven 640 (Affymetrix) rotating at 60 rpm at 45°C for 16 h. After hybridization, the arrays were washed on Genechip Fluidics Station 400 and scanned using Genechip Scanner 3000 (Affymetrix) according to the manufacturer's procedure.

Microarray output was visually inspected for physical damage and excessive background noise. Raw data of all probe sets on each array were normalized to a mean intensity of 2,000 according to 100 housekeeping genes before statistical analysis. Using GCOS (GeneChip Operation System, Affymetrix), absolute analysis was performed to determine whether the signal type of each transcript on the array was "present", "marginal" or "absent", and to calculate the raw signal representing the abundance of the tested transcript in each sample.

RNA interference

siRNA duplexes (si-CYPA, 5′-GCATACGGGTCCTGGC ATC-3′ [19]; si-CYPJ, 5′-CTGGAAGAGGAGGCAAC AG-3′) containing 3′dTdT over-hanging sequences were synthesized at Shanghai GeneChem Co.. A control nucleotide, si-control, was also purchased from GeneChem. The sequences of si-CYPA and si-CYPJ do not have significant identity to mRNA for *CYPJ* and *CYPA*, respectively. The concentration of si-RNA duplexes for transfection is 20 µM. The effect of si-CYPA and si-CYPJ on target gene expression was checked by western blot and RT-PCR, respectively.

RT-PCR/quantitative real-time RT-PCR

The primers for amplification were listed in Table 2. For RT-PCR analyses, cDNA was synthesized using 2 mg of total RNA. SuperscriptII reverse transcriptase (Gibco BRL) plus Oligo(dT)15 (Promega) was used according to the manufacturer's protocols. First-strand cDNA was subjected to RT-PCR amplification on FS-918 DNA Amplifier (Shanghai Fusheng Institute of Biotechnology). To optimize the cycle number, PCR amplifications were performed for 20–37 cycles (94°C 30 s, 63°C 30 s, 72°C 30 s). The products from each cycle were separated by on 2% agarose gel electrophoresis and the growth curve of the PCR products was made according to the amount of PCR products in different cycles. Then the optional cycle number was determined between the increasing logarithmic phase and plateau phase (28 cycles for all tested genes).

Quantification of genes was performed by SYBR Green I staining (Takara, Inc.) on an iCycler iQTM system (BioRad). To compare the relative levels of gene expression, we used cDNA from a normal adult brain (Clontech, Inc.) as standard. The optional conditions were as follows: 40 cycles of three-step PCR (95°C for 50 s, 63°C for 1 min, and 72°C for 30 s) after initial denaturation (95°C for 5 min). The real-time PCR reactions were performed in 25 µl volumes including 1 µl of cDNA template and each primer to a final concentration of 200 nM. Gene expression levels in each cDNA sample were normalized to the internal *GAPDH* levels. The experiments were carried out twice for each data point. For the negative controls, template cDNA was not added in to the mixture, and the PCRs were performed to avoid genomic DNA pollution.

Luciferase reporter assay

The MRP3 promoter-luciferase reporter plasmid MRP3-p-520 constructed in pGL3-basic vector was generously provide by Dr. Takeshi Uchiumi (University of Occupational and Environmental Health, Japan) [20]. The reporter construct was cotransfected into HEK-293 T cells with pCMV-CYPA, pCMV-CYPAm (R55A&F60A), pCMV-CYPJ, pCMV-CYPJm (R44A&F49A), or the control pCMV-HA vector alone using LipofectAMINE 2000 agents (Invitrogen). Thirty hours after transfection, cells lysates were prepared and luciferase activity was determined using Luciferase Assay System. The plasmid pRL-SV40 (Promega) encoding Renilla luciferase was used as the internal control in each transfection.

Analysis of drug sensitivity

The assay for drug sensitivity was modified from an existing protocol [21]. Drug sensitivity was analyzed using the MTS assay (Promega). SK-Hep1-pcDNA3.1, SK-Hep1-CYPA and SK-Hep1-CYPJ cells were seeded in triplicate at 1,000 cells/well in 96-well dishes in RPMI 1640 medium containing 10% FCS. The following day, we added taxol, doxorubicin (DOX), and vincristine (VCR) to the growth medium at various concentrations. Growth assays were performed after 72 h of incubation in the presence of drug.

Cellular accumulation of doxorubicin

The assay for cellular accumulation of doxorubicin was modified from previously published method [22]. Log-phase cells at a density of 1×10^6/ml in buffered RPMI 1640 supplemented with 10% FBS were treated with 0 uM, 1 uM or 4 uM DOX. After 3 h of treatment, the cells were centrifuged (1,000*g*) and washed twice with 5 ml of ice-cold PBS. The cell pellets following the final wash were re-suspended in PBS and then subjected to flow cytometry analysis (FACSCalibur, Becton Diction) at excitation and

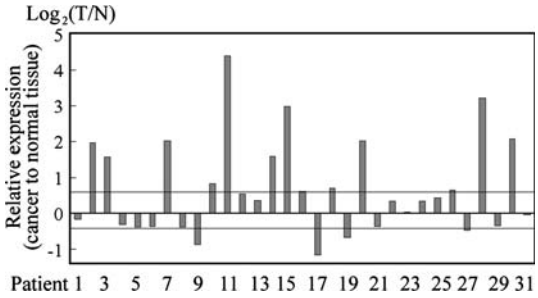

Fig. 1 Relative expression of *CYPA* in 31 pairs of HCC/adjacent non-cancerous tissues analyzed by realtime PCR. Logarithmic ratios of cancer to their corresponding non-cancerous tissues are shown. The *lines* indicate value of cancer/non-cancerous tissue for 1.5- and 0.75-fold

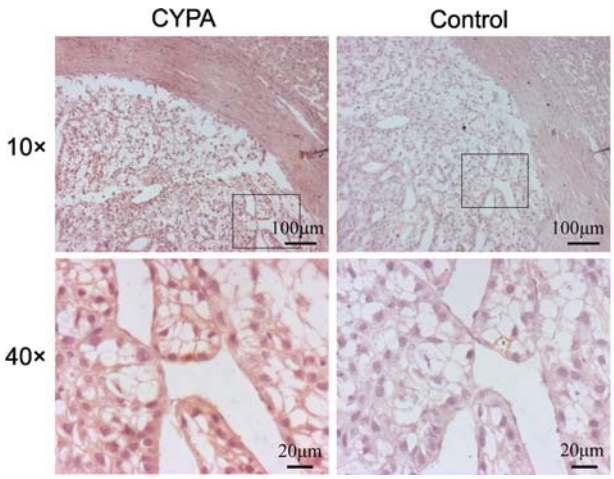

Fig. 2 Representive immunostaining of CYPA in human hepatocellular carcinoma. **a**, **b** showed that CYPA not only distributed in HCC, but in surrounding nonmalignant part of HCC. In HCC it mainly distributed in cytoplasm, cell membrane staining also observed. **c**, **d** is control: ommision of primary antibody as a control

emission wavelengths of 470 and 585 nm, respectively (controlled by CELLQUEST software); 10^4 cells were accounted and the average fluorescence level was used to indicate the cellular accumulation of DOX. The experiments were repeated three times.

Results

Up-regulation of *CYPA* in hepatocellular carcinoma tissues

We examined the expression of *CYPA* in 31 pairs of HCC/adjacent non-cancerous tissues by quantitative realtime RT-PCR. The expression value of *GAPDH* was used as internal control for normalization. The result revealed that *CYPA* mRNA was up-regulated in 13 out of 31 (41.9%) tumorous specimens for more than 1.5-fold compared with adjacent non-cancerous tissues (Fig. 1), which coincide with previous report [17]. The location of CYPA protein was also detected in HCC tissues using immunohistochemistry (Fig. 2). The demonstration for the specificity of the antibody is shown in supplemental figures. CYPA staining indicates its location both in nucleus and cytoplasm, and control staining without the first antibody shows no obvious signature.

Stable expression of CYPA and CYPJ in SK-Hep1 cells

In order to study the roles of elevated cyclophilin A in liver cells, we generated stably expressed CYPA in SK-Hep1 cells. Another member of cyclophilin family, *CYPJ*/*PPIL3* was also used to generate another stable cell line. The plasmids pcDNA3.1-myc, pcDNA3.1-CYPA and pcDNA3.1-CYPJ were transfected into SK-Hep1 cells. After G418 selection for 3 weeks, three cell lines SK-Hep1-pcDNA, SK-Hep1-CYPA, and SK-Hep1-CYPJ were obtained. The expression of CYPA protein is up-regulated in SK-Hep1-CYPA cells revealed by western blot (Fig. 3a). Increased *CYPA* mRNA expression in SK-Hep1-CYPA stable cell line and increased *CYPJ* mRNA expression in SK-Hep1-CYPJ stable cell line were also validated by real-time PCR (Fig. 3b).

Transcriptome modulated by CYPA

CYPA has been implicated to participate in many pathological processes, such as tumor, RA, and HIV infectious. The

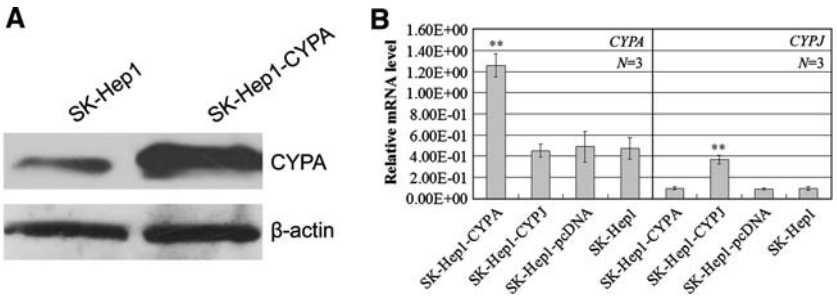

Fig. 3 Elevated expression of CYPA or CYPJ in stably transfected cells. **a** Increased intracellular CYPA expression in SK-Hep1-CYPA cells detected by western blot. Rabbit anti-CYPA polyclonal antibody was applied, and *β*-actin was used as an internal control (*bottom*). **b** The relative mRNA level of *CYPA* (*left figure*) and *CYPJ* (*right figure*) in SK-Hep1-CYPA, SK-Hep1-CYPJ, SK-Hep1-pcDNA, and wild type SK-Hep1 cells were detected by quantitative real-time RT-PCR. *GAPDH* was used as internal control for normalization. Overexpression of *CYPA* and *CYPJ* was found in SK-Hep1-CYPA cells and SK-Hep1-CYPJ cells, respectively

Table 1 Statistical catalogs of deregulated genes modulated by CYPA

Functional catalogs[a]	Up-regulated	Down-regulated
Transcriptional factor/regulator (168[b], 14.2%[c])	50	118
Protein kinase (40, 3.4%)	12	28
Protein phosphatase (15, 1.3%)	4	11
Cytokines and their receptors (42, 3.5%)	35	7
G protein/G protein coupled receptor (106, 8.9%)	60	46
Membrane proteins (113, 9.5%)	51	62
Cell cycle related gene (15, 1.3%)	5	10
Apoptosis related gene (15, 1.3%)	4	11
Matrix, cell structure (175, 14.8%)	107	68
Protease, peptidase (69, 5.8%)	47	22
Protein metabolism (82, 6.9%)	37	45
Metabolism (244, 20.6%)	137	107
Intracellular signal transduction (21, 1.8%)	10	11
Angiogenesis (6, 0.5%)	0	6
Others (74, 6.2%)	23	51

[a] The classification is performed based on functional annotation of each gene at Gene Ontology (http://www.geneontology.org)

[b] The number of genes detected per category

[c] The number of genes detected per category/the number of all deregulated genes

molecular mechanism of CYPA in such processes is under exploration. In order to systemically analyze the CYPA regulated genes, we compared the transcriptome between SK-Hep1-CYPA and SK-Hep1-pcDNA using oligo-microarray. In order to avoid variation in array experiments, we established stable cell lines on the same condition, and extracted the total RNA of these two stable transfected cells at the same time. Two test-chips were used to examine the quality of these RNA samples. Normalization of probe sets between experimental array and baseline array were carried out under manufacturers' suggests, and housekeeping or spike control transcripts were also used to control the signal of hybridization (see the Affimatrix website for more detailed information; http://www.affymetrix.com). Modulated transcripts were defined according to the following criteria: (1) the expression difference between SK-Hep1-CYPA and SK-Hep1-pcDNA cells is at least twofold and the signal is not AA; (2) if the change *P*-value is below 0.002, then the transcript expression in the experimental sample increases in contrast to the baseline sample; if the change *P*-value is higher than 0.998, the changing trend is considered as decreasing from the experimental sample to the baseline. The total probe sets is 54675 in HG-U133_Plus_2.0 oligo-chip. The number of 'Present' is 21,452 (39.2%), 'Absent' is 32,517 (59.5%), and 'Marginal' is 706 (1.3%). After statistical selection based on the upper two criteria, 2,715 transcripts were obtained (1,537 transcripts were increased in SK-Hep1-CYPA cell line and 1,178 transcripts with decreased expression value). Among them, 1,185 transcripts were functionally well-annotated genes (582 up-regulated and 603 down-regulated). The statistical catalogs of deregulated genes were listed in Table 1, and for more details please see the online supplemental tables (Table 2).

Twenty-eight selected cytokine related genes (including cytokines, cytokine receptors, and signaling transducers that regulate the expression of cytokine genes) are listed in Table 3, in which many differentially expressed cytokines

Table 2 Primer sequences for RT-PCR/quantitative real-time RT-PCR

Gene name	Forward primer (5′–3′)	Reverse primer (5′-3′)
GAPDH	CACTCCTCCACCTTTGACG	ACCACCCTGTTGCTGTAGC
CYPA_CDS	AACTTCATCCTAAAGCATACGG	TTGCCATCCAACCACTCAG
CYPJ_CDS	CATCACCTATGGCAAACAGC	TGGCAACTTCTCCAACTCATC
IL1A	TGACTGCCCAAGATGAAGACC	TCCCAGAAGAAGAGGAGGTTG
IL1B	TGCGTGTTGAAAGATGATAAG	TTGGGGAACTGGGCAGAC
IL6	CTCCAGAACAGATTTGAGAGTAGTG	TTGTGGTTGGGTCAGGGGTG
IL8	GGGTTGTGGAGAAGTTTTTG	GTTTCACTGGCATCTTCACTG
IL12A	TGCAAAGCTTCTGATGGATC	CGGTTCTTCAAGGGAGG
IL24	GCAACCCAGTCAAGAAAATG	CCAGGTCAGAAGAATGTCC
STAT1	CTGAGGAGTTTGACGAGGTG	ACAGAGTAGCAGGAGGGAATC
STAT2	CCAGACCAAGGACCTGTATCAC	TGGGTCACCATTCGGCATG
MRP2	TTATCCCACGAAGTGACAGAG	GATTTCCGAAGCAGAGCC
MRP3	ATGTCCGCAGAATGGACTTG	CACCACTTGGGGATCATTTC
MRP4	GAACACCATTATTGACAGCG	AGCAAAACATACGGCTCATC
MGST1	GCCAATCCAGAAGACTGTGTAG	TTTCAAGGTCATTCAGGTGG
GSTZ1	TACTGTGTAGGAGACGAGGTGAC	AGCTGATGGTAGGGTAGGGG

Table 3 Deregulated cytokine related genes revealed by microarray

Symbol	GenBank ID	Gene discription	SLR[a]
Interleukin			
IL1A	NM_000575	Interleukin 1, alpha	3.2
IL1B	NM_000576	Interleukin 1, beta	4.4
IL6	NM_000600	Interleukin 6 (interferon, beta 2)	4.4
IL8	NM_000584	Interleukin 8	5.9
IL11	NM_000641	Interleukin 11	1.8
IL12A	NM_000882	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	1.2
IL24	NM_006850	Interleukin 24	4.7
IL32	NM_001012631	Interleukin 32	1.8
Chemokine			
CXCL1	NM_001511	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	5.1
CXCL2	NM_002089	Chemokine (C-X-C motif) ligand 2	5.3
CXCL3	NM_002090	Chemokine (C-X-C motif) ligand 3	4.8
CCL5	NM_002985	Chemokine (C-C motif) ligand 5	1.8
CCL26	NM_006072	Chemokine (C-C motif) ligand 26	−1.3
CKLF	NM_001040138	Chemokine-like factor	−1.5
Other cytokines			
CSF2	NM_000758	Colony stimulating factor 2 (granulocyte–macrophage)	5.3
GFER	NM_005262	Growth factor, augmenter of liver regeneration (ERV1 homolog, *S. cerevisiae*)	−1
FGF2	NM_002006	Fibroblast growth factor 2 (basic)	1.7
FGF5	NM_004464	Fibroblast growth factor 5	−1.7
GDF9	NM_005260	Growth differentiation factor 9	1
GDF15	NM_004864	Growth differentiation factor 15	1.6
Receptor			
IL1R1	NM_000877	Interleukin 1 receptor, type I	1.9
IL10RB	NM_000628	Interleukin 10 receptor, beta	1.5
IL13RA2	NM_000640	Interleukin 13 receptor, alpha 2	5
IL27RA	NM_004843	Interleukin 27 receptor, alpha	1.1
IL31RA	NM_139017	Interleukin 31 receptor A	1.2
CXCR4	NM_001008540	Chemokine (C-X-C motif) receptor 4	−3.4
EGFR	NM_005228	Epidermal growth factor receptor	−3
TNFRSF1B	NM_001066	Tumor necrosis factor receptor superfamily, member 1B	1.4
FGFR2	NM_000141	Fibroblast growth factor receptor 2	1.2
IGF2R	NM_000876	Insulin-like growth factor 2 receptor	1.1
IFNGR2	NM_005534	Interferon gamma receptor 2 (interferon gamma transducer 1)	1
Signaling transducer			
SOCS1	NM_003745	Suppressor of cytokine signaling 1	1.6
STAT1	NM_007315	Signal transducer and activator of transcription 1, 91 kDa	1
STAT2	NM_005419	Signal transducer and activator of transcription 2, 113 kDa	1.4

[a] SLR Signal log ratio

are coincides with other's results, such as IL-1β and IL-8 [10]. We also found 12 drug metabolism and drug transport related genes up-regulated by CYPA over-expression (Table 4). Some of these genes may contribute to drug resistance, such as *IL6* [23], *MRP2* [24], *MRP3* [25], *MGST1* [26] and *GSTZ1* [27]. These results suggest that CYPA over-expression may lead to drug resistance, which result in a bad outcome of chemotherapy.

Table 4 Deregulated genes related to drug metabolism and transport

Symbol	GenBank ID	Gene description	SLR
Multidrug resistance associated protein			
ABCC2	NM_000392	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	1.3
ABCC3	NM_003786	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	6.1
ABCC4	NM_005845	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	−1.2
Glutathione *S*-transferase			
MGST1	NM_020300	Microsomal glutathione *S*-transferase 1	1.1
GSTZ1	NM_001513	Glutathione transferase zeta 1 (maleylacetoacetate isomerase)	1.1
Cytochrome P450 family member			
CYP1B1	NM_000104	Cytochrome P450, family 1, subfamily B, polypeptide 1	1.3
CYP4A11	NM_000778	Cytochrome P450, family 4, subfamily A, polypeptide 11	1.2
CYP2R1	NM_024514	Cytochrome P450, family 2, subfamily R, polypeptide 1	1.1
Metallothionein			
MT1M	NM_176870	Metallothionein 1M	1.3
MT1F	NM_005949	Metallothionein 1F (functional)	1.3
MT1G	NM_005950	Metallothionein 1G	1.1
MT1H	NM_005951	Metallothionein 1H	1.1
MT1X	NM_005952	Metallothionein 1X	1.1

Fig. 4 Validate the results of microarray by RT-PCR. The expression of several cytokine or drug resistance related genes in SK-Hep1-pcDNA, SK-Hep1-CYPA and SK-Hep1-CYPJ cells were detected. *GAPDH* were used as internal control

Validate the microarray results

We validated the expression of 13 genes in cell lines SK-Hep1-CYPA, SK-Hep1-CYPJ and SK-Hep1-pcDNA using RT-PCR, including *IL1A*, *IL1B*, *IL6*, *IL8*, *IL12A*, *IL24*, *STAT1*, *STAT2*, *MRP2*, *MRP3*, *MRP4*, *MGST1*, and *GSTZ1*. As shown in Fig. 4, the expression of *IL1A*, *IL1B*, *IL6*, *IL8*, *IL12A*, *IL24*, *STAT1*, *STAT2*, *MRP2*, *MRP3*, and *MGST1* in SK-Hep1-CYPA cells is significantly higher than that in the control cells. For comparison, the expression of *IL6*, *IL8*, *STAT1*, *MRP2*, and *MGST1* is also significantly up-regulated by CYPJ over-expression.

We also employed quantitative real-time RT-PCR to detect the expression of these 13 genes. At the mean time, we decreased the expression of CYPA or CYPJ by RNAi technology, and compared the expression of these genes to control cells. The efficiency of CYPA and CYPJ siRNAs to knockdown the expression of target gene in SK-Hep1 cells was checked (Fig. 5). As shown in Table 5, 12 of the tested genes were up-regulated by more than twofold in SK-Hep1-CYPA cells expect *MRP4*, which is up-regulated by 1.3-fold and it was down-regulated in microarray results. For CYPJ stable cell line, 11 genes are up-regulated by more than twofold while *IL12A* and *MGST1* is elevated for 1.8- and 1.4-fold, respectively. In RNAi experiments, we found that the expression of *IL1A*, *IL1B*, *IL6*, *IL8* and *MRP3* can be decreased by more than 30% after CYPA knockdown, whereas *IL1A*, *IL8* and *IL24* exhibited decreased expression by more than 30% after CYPJ knockdown. Taken together, the up-regulation of *IL1A*, *IL1B*, *IL6*, *IL8* and *MRP3* by CYPA can be validated in both experiments, and the up-regulation of *IL1A*, *IL8* and *IL24* by CYPJ can be confirmed in both experiments.

In all drug resistance-related genes, the expression of MRP3 is the greatly modified after CYPA over-expression. In order to further characterize the regulation of MRP3 promoter by CYPA, we applied an MRP3 promoter-luciferase reporter plasmid in the study. CYPJ and the PPIase

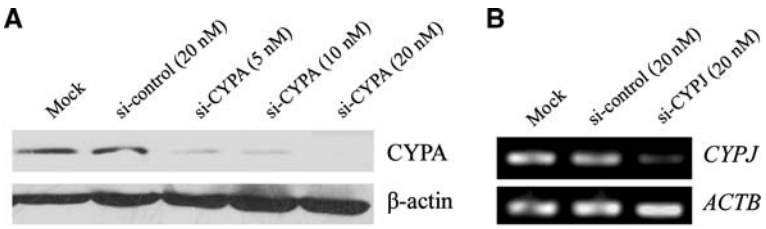

Fig. 5 Knockdown the expression of endogenous CYPA and *CYPJ*. **a** For CYPA, the efficiency of si-CYPA was checked at protein level by rabbit polyclonal anti-CYPA antibody. *β*-actin was used for normalization. *Mock* indicate cells do not transfected with any siRNA complexes; si-control was transfected at 20 nM, and si-CYPA was transfected at different levels. **b** For *CYPJ*, the efficiency of si-CYPJ was checked at mRNA level by RT-PCR. *ACTB* mRNA was used for normalization. *Mock* indicate cells do not transfected with any siRNA complexes; si-control and si-CYPJ were transfected at 20 nM

Table 5 Validation of microarray results by quantitative real-time RT-PCR

Symbol	Deregulated relative mRNA level (fold change)			
	Operate with CYPA		Operate with CYPJ	
	Over-expression[a]	RNAi[b]	Over-expression[c]	RNAi[d]
IL1A	**17.8**[e]	**0.284**	**9.2**	**0.287**
IL1B	**148.3**	**0.568**	14.7	0.798
IL6	**141.2**	**0.248**	7.1	0.749
IL8	**411.3**	**0.643**	**22.1**	**0.698**
IL12A	3.2	0.912	1.8	0.912
IL24	939.8	0.759	**20.8**	**0.659**
STAT1	2.8	0.730	2.1	0.799
STAT2	6.3	0.914	6.1	0.985
MRP2	2.5	0.712	3.2	1.1
MRP3	**140.8**	**0.462**	3.7	0.964
MRP4	1.3	0.870	2.4	1.6
MGST1	4.6	1.0	1.4	0.998
GSTZ1	2.1	1.0	2.5	1.0

[a] The value is calculated by comparing the relative mRNA level for each gene between cell line SK-Hep1-CYPA and control cell SK-Hep1-pcDNA

[b] The value is calculated by comparing the relative mRNA level for each gene between SK-Hep1 cells transfected with si-CYPA and si-control

[c] The value is calculated by comparing the relative mRNA level for each gene between cell line SK-Hep1-CYPJ and control cell SK-Hep1-pcDNA

[d] The value is calculated by comparing the relative mRNA levels for each gene between SK-Hep1 cells transfected with si-CYPJ and si-control

[e] The value bolded means this gene is up-regulated in CYPA/CYPJ over-expression experiment for more than twofold, and is down-regulated in CYPA/CYPJ RNAi experiment for more than 30% at the same time

mutants of CYPA/CYPJ were also used in the experiment for comparison. In HEK-293T cells, CYPA and CYPJ can promote the transcription of MRP3 by tenfold and fivefold, respectively (Fig. 6a). When we co-transfected different amount of CYPA with MRP3-Luc reporter plasmids, the concentration dependent activation is obvious (Fig. 6b). The amino acid F55 and R60 are important to CYPA, and mutation at any site will absolutely disrupt the PPIase activity of CYPA [28]. We mutated these two amino acids of CYPA to Ala, and by sequence alignments, we found and subsequent mutated the corresponding sites of CYPJ to Ala. The PPIase activity of CYPA mutant is completely disrupted, and the PPIase activity of CYPJ mutant is decreased by approximately 68% (data not shown). As shown in Fig. 6a, the mutations in CYPA and CYPJ evidently weaken their activities to stimulate the transcription of MRP3 promoter ($P < 0.01$ between CYPA and CYPAm; $P < 0.05$ between CYPJ and CYPJm). These results indicate that the regulation of MRP3 promoter by CYPA and CYPJ is some what dependent on their PPIase activities.

Roles of CYPA and CYPJ in drug resistance

To evaluate roles of CYPA and CYPJ in drug resistance, the drug sensitivities of these two stably transfected cells (SK-Hep1-CYPA and SK-Hep1-CYPJ) were compared with control cells. The drug sensitivities of these cell lines were analyzed by 3-day cell growth assay. Increased resistance was observed for anticancer drug DOX and VCR (Fig. 7). The accumulation of DOX was also observed reduced in SK-Hep1-CYPA and SK-Hep1-CYPJ cells (Fig. 8). At the concentration of 1 μM, SK-Hep1-CYPA and SK-Hep1-CYPJ accumulate 10 and 12% less DOX than control cells (P-value <0.001), while at concentration of 4 μM, no obvious differences were found in these three cell lines. These data suggest that the elevated expression of CYPA in liver cells contributes to drug resistance, and may contribute to clinical resistance to anti-cancer drugs in HCC chemotherapy.

Discussion

The completion of human genome project (HGP) has resulted in a large amount of sequence information.

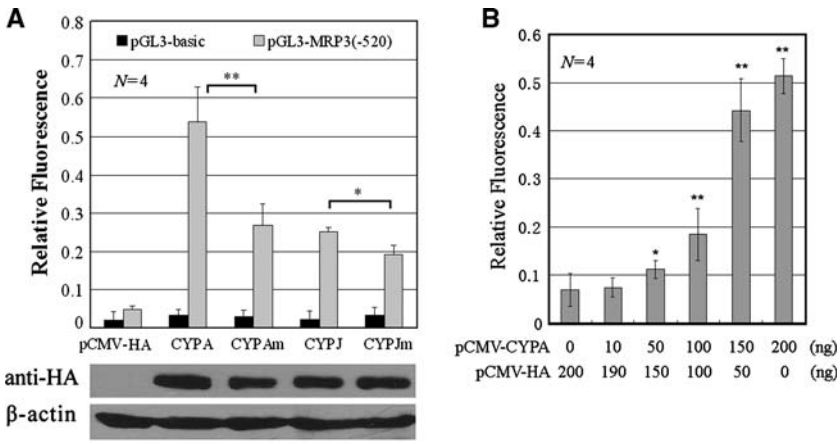

Fig. 6 Activation of MRP3 promoter (MRP3-p-520) by CYPA and CYPJ. **a** CYPA and CYPJ can activate the transcription of MRP3 promoter in a PPIase dependent manner. Each transfection was detected by western blot. **b** Activation of MRP3 promoter by different amount of CYPA transfection. *Indicates $p < 0.05$, **indicates $p < 0.01$

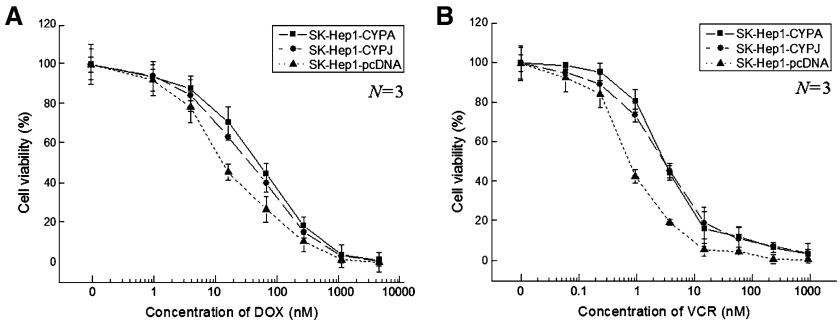

Fig. 7 Sensitivity of SK-Hep1-pcDNA, SK-Hep1-CYPA and SK-Hep1-CYPJ cells to doxorubicin **a** and vincristine **b** revealed by 3-day cell growth assay

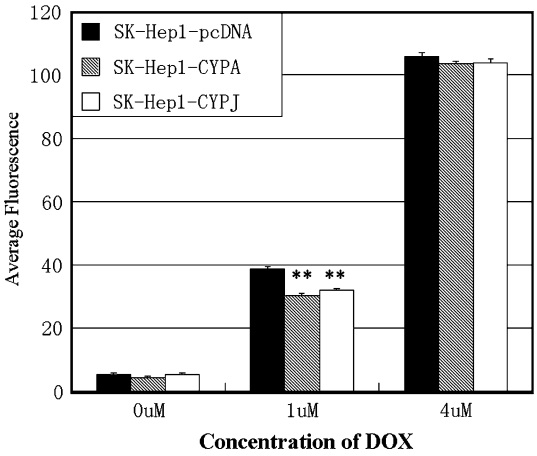

Fig. 8 Accumulation of doxorubicin in SK-Hep1-pcDNA, SK-Hep1-CYPA and SK-Hep1-CYPJ cells. The experiment procedure is described at Materials and Methods section. **Indicates $p < 0.01$

Thousands of novel genes were predicted and subsequently isolated. For most of these genes, their functions remain unknown. On the other hand, there are only about 20,000–25,000 protein-coding genes in human genome [29], so each gene may contain multiple functions. In the post-genome era, it is a challenge to predict the function of novel genes [30, 31]. The yeast two-hybridization is a commonly used method to search for binding partners of a given protein [32]. Protein array and mass spectrometry are recently developed techniques to detect protein–protein interaction [30]. RNA interference (RNAi), which gained the Nobel Prize in 2006, was successfully used in mammalian cells to inhibit endogenous gene expression [33].

Cyclosporin A (CsA) is a clinically important immunosuppressive drug widely used to prevent graft rejection after organ or bone marrow transplantation [34]. Recent years, several reports show that CsA can reverse drug resistance when combined with some anti-caner agents. Some mechanisms were put forward, such as modulating the activity of MRP1 [35] and Pgp [36], and stimulation of MAPK [37], AKT [38] and NFκB [39] cell signaling pathways. One mechanism of drug resistance is reducing intracellular drug accumulation, which in turn weaken the cytotoxicity of anti-cancer drugs. ABC transporters are good examples for this kind of resistance [40]. In the present work, we found that CYPA can promote the expression of many ABC transporters and several cytokines related to inflammation. Furthermore, we found that the transcriptional regulation of MRP3 promoter is PPIase dependent (Fig. 6). CYPA has been previously shown induced by hypoxia stress to protect cells against oxidative stress-induced apoptosis [41, 42], and related to Rb [43], NFκB [44] and Itk signal pathways [45]. So we speculate that the activity for CsA to reverse drug resistance may partially due to the inhibition of the activity of CYPA for CsA is a potent PPIase inhibitor. Subsequent experiments indicate that over-expression of CYPA leads to DOX and VCR

resistance in SK-Hep1 cell line in drug sensitivity assay (Fig. 7) and drug accumulation assay (Fig. 8). These findings suggest that CYPA may assist tumor cells to live in disadvantageous environments such as hypoxia and chemotherapy, and subsequently play roles in the process of carcinogenesis by this way.

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