Screening on human hepatoma cell line HepG-2 nucleus and cytoplasm protein after CDK2 silencing by RNAi

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Abstract The activation of phase-specific cyclin-dependent kinases is associated with ordered cell cycle transitions. Among the mammalian Cdk2 is essential for liver cancer cell proliferation. The related cycling protein CDK2 was analyzed by 2D-gel and MALDI-TOF/TOF MS mass assay in liver cancer cells, which CDK2 was silenced. The results showed four significantly different spots in cell ribonucleoprotein (similar to ribosomal protein S12, chaperonin 10-related protein, beta-actin and zinc finger protein 276) and four in plasmotin (aldolase A protein, hCG,

anonymous protein and tubulin, gamma complex associated protein 2). In the plasmotin, aldolase A catalyzes the production of tubulin and actin. Together they regulate the cell cycle and arrest the cell in the S phase. In the cell ribonucleoprotein, proteins with homology to ribosomal protein S12 and chaperonin 10 play a similar role in cell cycle regulation.

Keywords CDK2 · 2D-gel · Proteome · HepG2 cells

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Introduction

The G1 cyclin-dependent kinases (Cdks), a family of serine/threonine protein kinases, control the onset of the major cell cycle events, such as DNA synthesis and mitosis (Wadler 2001). Metazoan cells have multiple Cdks and cyclins that are temporally regulated. In normal cell cycles, cyclin-dependent kinase-2 (Cdk2) pairs with E- and A-type cyclins during S and G2 phase, and cyclin-independent kinase-1 (Cdk1) pairs with A- and B-type cyclins during G2 and M phase (Li et al. 2010; Hinchcliffe and Sluder 2002). In the cell cycle, the importance of Cdk regulated transitions was first suggested by studies in which the introduction of inhibitory antibodies or the expression of dominant negative mutants or small molecule inhibitors caused phase-specific cell cycle arrest (Golsteyn 2005). Recent genetic studies, however, have called into question the requirement for multiple Cdks (Golsteyn

2005; Copeland et al. 2010). In human cells, RNAi-mediated depletion of Cdks showed that Cdk2 is dispensable for cell cycle progression (Copeland et al. 2010).

A previous study, the Cdk4/Cdk6-cyclin D pathway has been studied. In this study, we mainly investigated on the Cdk2-cyclin E function in liver cancer cells (Shimura et al. 2012; Bienvenu et al. 2001). In somatic cells, the G1/S transition is governed by Cdk2-cyclin E complexes through their action on several targets (Chung and Bunz 2010). Harvey et al. (2000) examined Cdk2 activity in HeLa cells that entered apoptosis after treatment with staurosporine. After expression of Cdk2D145N, a variant that is catalytically inactive, they noted a reduced number of cells that displayed condensed chromatin after treatment with staurosporine (Adon et al. 2010).

Cyclin-dependent kinases-2 activities are positively regulated by association with different cyclins which are temporally expressed at specific phases of the cell cycle (Hinchcliffe and Sluder 2002). The detailed mechanism of CKD2 regulation by cyclins in liver cancer cells is still unclear. In this study, CDK2 was inhibited in the liver cancer cells HepG2, the cyclins and cell proliferation were assayed and the subcellular structure proteins were investigated by 2D-image master-MS. This process provides a novel way to study CDK2 and how it affects cell cyclins and cell proliferation.

Materials and methods (M&M)

Cell culture

The liver cancer cell line HepG2 (Chinese Academy of Sciences Cell Bank, Shanghai, China) was grown as a monolayer in RPMI-1640 (Hyclone, Logan, UT, USA) containing 10 % fetal bovine serum (Hyclone). Cells were maintained at 37 °C in a humidified incubator under a 5 % CO₂ atmosphere.

RNA interference

Cdk2 small interfering RNAs (siRNAs) was purchased from Santa Cruz Biotechnology, Inc Santa Cruz, CA, USA). The two siRNA duplex sequences targeting the CDK2 mRNA are NM_001122742.1 (sense, 5'-GATCCGGTGTACCCAGTACTGCCATT

CAAGACGTGGCAGTACTGGGTACACCTTTTTTGTCGACA-3'; antisense, 3'-GCCACATGGGTCTGACGGTAAGTTCTGCACCGTCATGACCCATGTGGAAAAACAGCTGTTCTGA-5') and all siRNAs were prepared using a transcription-based method with the Silencer siRNA construction kit (Integrated DNA Technologies, San Diego, CA, USA) according to the manufacturer's instructions. Three days after the addition of complete medium, cell lysates were prepared for appropriate assays.

Transfection of siRNA

siRNA transfection was performed according to the lipofectamine protocol supplied by Invitrogen (Carlsbad, CA, USA). Briefly, 1×10^5 cells were seeded into six-well plates containing an antibiotic-free medium and incubated overnight at 37 °C. For each well, 5 µL siRNA was mixed with 125 µL OPTI-MEM I (Invitrogen). The mixture was then combined with a solution of 5 µL lipofectamine (Invitrogen) in 125 µL OPTI-MEM I. After a 20-min incubation period at room temperature, the mixture was applied to the cells in an appropriate volume of OPTI-MEM I so as to achieve a final concentration of 100 nM for each siRNA. After incubation for 6 h at 37 °C, RPMI-1640 supplemented with serum was added to the wells and cells were cultured for an additional 24 h at 37 °C before analysis.

Western blotting

Cells were harvested 48 h after transfection, washed with 37 °C PBS, and lysed with a phenylmethanesulfonyl fluoride lysis buffer (Invitrogen). After centrifugation at 13,200g for 30 min, the supernatant was analyzed for protein content using the bicinchoninic acid reagent (Sigma, St. Louis, MO, USA). Total protein (50 µg) from each sample was electrophoresed on a 10 % SDS-PAGE gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with PBS containing 5 % fatfree milk (BD, San Jose, CA, USA) and 0.1 % Tween-20 (Sigma) for 30 min at room temperature, and were then incubated with primary anti-CDK2 antibody (Millipore) for at least 1 h at room temperature, or overnight at 4 °C. The membranes were washed three times with PBS containing 0.1 % Tween 20, incubated

with peroxidase conjugated secondary antibodies (Millipore USA), and developed using the ECL reagent (Pierce Biotechnology Inc., Rockford, IL, USA).

Flow cytometry analysis (FCM)

Cell-cycle distribution was evaluated by 5-bromo-2'-deoxyuridine (BrdU) incorporation and propidium iodide staining. Cells were pulsed with 10 μ M BrdU (Sigma-Aldrich) for 30 min, trypsinized (0.5 % trypsin–ethylenediaminetetraacetic acid (Taneja and Mande 2001); Gibco-Invitrogen, Carlsbad, CA, USA) to obtain a single cell suspension, washed twice in PBS with 1 % FBS, and resuspended in PBS. The cells were then fixed in ice cold 70 % ethanol. After rehydration in PBS with 1 % FBS (Gibco), cells were incubated in 2 M HCl with 0.5 % (v/v) Triton X-100 (Sigma) for 30 min at room temperature. Following neutralization with 0.1 M $\text{Na}_2\text{B}_4\text{O}_7$, cells were collected by centrifugation at 2,000g for 5 min and washed with PBS with 1 % FBS and 0.5 % (v/v) Tween-20. They were then stained with anti-BrdU fluorescein isothiocyanate (FITC)-labeled antibody (1:20; Roche Diagnostics, Mannheim, Germany) for 30 min at room temperature in darkness. The cells were then washed with PBS with 1 % FBS and 0.5 % (v/v) Tween-20 and incubated in 1.1 % sodium citrate with 5 ng/ μ L ribonuclease A (DNA Lego Ribonuclease A; Top-Bio, Prague, Czech Republic) and 60 μ g/ μ L propidium iodide (Sigma-Aldrich) for 30 min at 37 °C in the dark. Cells were analyzed by flow cytometry on the Cytomics FC 500 machine using the CXP software (Beckman Coulter, Fullerton, CA, USA) following standard procedures. Analysis by Multi-Cycle (Phoenix Flow Systems, San Diego, CA, USA) was applied to assess cell-cycle distribution.

Ribonucleoprotein and plasmosins obtained

Ribonucleoprotein extract of asynchronously proliferating human HepG2 cells was obtained from a kit (Pierce Biotechnology Inc., Rockford, IL, USA). Extract was diluted in replication buffer (20 mM K-HEPES pH 7.8, 100 mM K-acetate, 1 mM DTT, 1 mM EGTA). The plasmosins extracts were prepared as above using a kit (Pierce Biotechnology Inc.).

Two-dimensional gel electrophoresis

Cell total protein was solubilized and denatured in 350 μ L of 2-DE sample buffer 7 M urea, 2 M thiourea, 2 % v/v Triton X-100, 65 mM DTT, 0.5 % v/v Pharmalyte 3–10, and 10 % isopropanol (Pierce). Briefly, samples were applied to 18 cm pH3–10 IPG gel strips (Bio-Rad, Richmond, CA, USA), and submitted to isoelectrofocusing (IEF) using an Ettan IPGphor3 Unit (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. A voltage of 30 V was applied to the strips for 6 h, followed by 500 V for 1 h, 1,000 V for 1 h, and then 8,000 V for 4 h, with a maximum current of 50 μ A per strip, as described by Costa et al. (2011). After focusing, the proteins on the strips were reduced, alkylated and separated by 12 % SDS-PAGE on a Protean II system (Bio-Rad, USA). After silver-staining, the gels were either stored in 1 % acetic acid or dried in 1:5:30 acetic acid/glycerol/methanol (v/v/v).

Image capture and processing

Gels were visualized with colloidal Coomassie staining following an improved blue silver method. Briefly, a gel was fixed for 1 h initially in a fixation solution containing 40 % (v/v) methanol and 10 % (v/v) acetic acid, followed by four Milli-Q water washes (15 min each). Then the gel was stained overnight in the working colloidal “blue silver” solution with 0.12 % Coomassie Brilliant Blue G-250 (Sigma), 10 % ammonium sulfate (Sigma), 10 % phosphoric acid (Sigma), and 20 % methanol (Sigma). Subsequently, the gels were destained for 2 h via six Milli-Q water washes (20 min each).

Gels were scanned using a Gel-documentation system on a GS- 670 Imaging Densitometer (Bio-Rad). Gels were analyzed with the Image Master 2D Elite (Bio-Rad). Spot intensities were normalized with total valid spot volume in order to minimize the non-expression related variations in spot intensity and hence accurately provide semiquantitative information across different gels. Differences of ≥ 1.5 in expression (ratio %V) between matched spots were considered significant when a spot group passed statistical analysis (*t* test, *p* < 0.05).

Protein identification by mass spectrometry

Differentially expressed protein spots were manually excised from 2-DE gels, and washed sequentially with

water and 50 % acetonitrile (ACN) in 25 mM ammonium bicarbonate, until they were completely destained. After dehydration with 100 % ACN, the dry gel pieces were digested by adding 10.0 ng/ μ L trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate overnight at 37 °C.

For MALDI-TOF/TOF MS analysis, 2 μ L of the digest peptide was mixed with 1 μ L of matrix (100 mM α -cyano-4-hydroxy-cinnamic acid in 50 % acetonitrile (ACN) and 0.1 % trifluoroacetic acid Sigma) on a target plate. MALDI-TOF MS and TOF-TOF tandem MS were performed on a 4,800 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA), and data were acquired in the positive MS reflector mode with a scan range of 900–4,000 Da, and five monoisotopic precursors ($S/N > 200$) were selected for MS/MS analysis. For interpretation of the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm. One missed cleavage site was allowed for trypsin digestion, cysteine carbamidomethylation was assumed as a fixed modification, and methionine was assumed to be partially oxidized. Results with CI % (Confidence Interval %) values greater than 95 % were considered to be a positive identification. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (<http://www.geneontology.org/>).

Results

Down regulation of CDK2 protein expression in HepG2 cells after transfection with siRNA

The cells positively stained by the anti-CDK2 antibody accounted for 95–99 % of the total cell population. The control group showed strongly positive cytoplasmic expression of CDK2 (dark brown-yellow), while the CDK2-siRNA group showed weakly positive cytoplasmic expression (light brown-yellow). Western blotting showed that the CDK2 siRNA-transfected cells expressed significantly less CDK2 than the control groups (Fig. 1).

Fig. 1 siRNA-mediated down-regulation of Cdk2. Cells were transfected with cdk2-siRNA vector or control siRNA. Positive cells were selected by G418 as described ‘Experimental Procedures’. As compared to control, cells with the cdk2-siRNA vector reduced the cdk2 protein level by 99 % ($n = 5$). * $P < 0.05$ versus control

CDK2 inhibit HepG2 in G1—S phase

Using FCM, cell cycling was observed. In the CDK2-siRNA group, there were more cells in G1 phase. This indicated that CDK2 expression was affected in the G1—S phase (Fig. 2).

2-DE maps and protein identification by MALDI-TOF/TOF MS between CDK2-siRNA group and control group

Approximately 300 2-DE spots were detected on each gel after Coomassie brilliant blue G-250 staining, with 95 % of the spots matched on paired gels in cell ribonucleoprotein. Four protein spots were found to be significantly different (threefold or higher) in spot intensity, and they were successfully identified by MALDI-TOF/TOF MS (Fig. 3, Table 1). Approximately 100 2-DE spots were detected on each gel, with 90 % of the spots matched on paired gels in plasmosin. Four protein spots were found to be significantly different (threefold or higher) in spot intensity, and they were successfully identified by MALDI-TOF/TOF MS (Fig. 4, Table 2).

Discussion

CDK2 together with cyclin E can cause the cells to remain in the S phase. Previously, the function of

Fig. 2 CDK2 inhibits HepG2 in G1–S phase. Cells were transfected with cdk2-siRNA vector or control siRNA. Positive cells were selected by G418 as described in ‘Experimental Procedures’. Cell numbers of the cdk2-siRNA group **a** were

higher in G1 phase than in the control group (**b**). **c** Percentage of cells of cdk2-siRNA group and control group in G1 phase. * $P < 0.05$ versus control

cyclin E was silenced, and it was shown that the proliferation activity of HepG2 changed. In addition, the different proteins, detected by 2-DE maps and identified by MALDI-TOF/TOF MS, were also expressed in the CDK2 silenced HepG2 (Wang and Liu 2008). In the CDK2-siRNA group, there was a higher number of cells in the G1 phase (Xu et al. 2010). CDK2 can promote the cell to transition from the G1 phase to the S phase. During this process, many related proteins participate. By 2-D gel electrophoresis, many different protein spots can be distinguished between the CDK2-siRNA group and the control group. However there were only four spots with significantly different expression in the cell ribonucleoprotein (with homologies to ribosomal protein S12, chaperonin 10-related protein, beta-actin and zinc finger protein 276, respectively) and four in the

plasmosin extract (with homologies to aldolase A protein, hCG, anonymous protein and tubulin, gamma complex associated protein 2, respectively).

Ribosomal protein S12 is one of the small subunits in the ribosomal protein. It can regulate the accuracy and rate of translation (Mariottini et al. 1999). In HepG2 cells, when the CDK2 gene was silenced, an increase in a S12-like ribosomal protein was seen (Maisnier-Patin et al. 2007). This may have led to a decrease in the speed of cell translation, and this may have resulted in the arrest of the cell cycle in the S phase (Mariottini et al. 1999). When the *Cdk2* gene was silenced, the cells became stressed and the heat shock protein Chaperonin 10 was overexpressed (Sakane et al. 2004). This indicated that nuclear protein expression was largely and complexly changed in CDK2 silenced HepG2. Many proteins with

Fig. 3 Representative overview of 2-DE maps in cell ribonucleoprotein. (a) Cdk2-siRNA group (b) control group (c, d) significantly different spots observed between the Cdk2-siRNA group and the control. *Blue circle* The protein spots do not fully match between the control and the siRNA group in the nucleus. *Green circle* The proteins in the control group are three times higher than in the siRNA group in the nucleus. The protein spot marked by the green circle (c, d) corresponds to that marked by a red circle in the original electropherogramme (b)

Table 1 Differentially expressed cell ribonucleoproteins identified by MALDI-TOF/TOF MS

Spot	Accession	Score	pI	Mr($\times 10^3$)	Coverage (%)	Protein name
1.	gil51468838	65	6.43	14.931	45	Similar to ribosomal protein S12
2.	gil14250401	54	5.56	41.321	26	Actin, beta
3.	gil119587097	48	9.61	46.009	22	Zinc finger protein 276
4.	gil4028622	65	8.98	10.289	45	Chaperonin 10-related protein

homologies to chaperonin 10 were overexpressed and this may have also contributed to the arrest of the cell cycle in the S phase (Brown et al. 2005).

This changes also took place in plasminogen. In this study, when CDK2 was silenced, the aldolase A

protein, hCG, anonymous protein and tubulin, gamma complex associated protein 2 were expressed at significantly higher levels. hCG can block apoptosis in somatic cells (Cole 2007; Cole and Khanlian 2007). This may have contributed to keeping cells in S phase.

Fig. 4 Representative overview of 2-DE maps in plasmosin. (a)cdk2-siRNA group (b) control group; (c, d) significantly different spots observed between the Cdk2-siRNA group and the control. *Green circle* The protein in the control group are three

times higher than in the siRNA group in the cytoplasm. The protein spot marked by the green circle (c, d) corresponds to that marked by a red circle in the original electropherogramme (b)

Table 2 Differentially expressed cell plasmosins identified by MALDI-TOF/TOF MS

Spot	Accession	Score	pI	Mr($\times 10^3$)	Coverage (%)	Protein name
1.	gil10437371	70	8.97	25.782	28	Unnamed protein product
2.	gil28595	98	8.76	11.987	80	Aldolase A protein
3.	gil62898904	71	6.38	103.011	23	Tubulin, gamma complex associated protein 2
4.	gil119607042	36	8.59	7.898	52	hCG

Aldolase A catalyzes the cleavage of fructose 1,6-phosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate in the glycolytic pathway (Ueno et al. 2010). Besides its glycolytic role, aldolase bundles microtubules by tethering the subunit of tubulin and cross-linking actin filaments (Buscaglia et al. 2003). Tublin is one of the microtubule associated proteins. In association with the microtubules, it regulates the cycling of cells.

In conclusion, the cycling protein CDK2 is related to the ribonucleoproteins of the cell (similar to ribosomal protein S12, chaperonin 10-related protein, beta-actin and zinc finger protein 276) and the plasmosins of the cell (aldolase A protein, hCG, anonymous protein and tubulin, gamma complex associated protein 2). Aldolase A is involved in the tethering of tubulin and cross-linking of actin filaments. Together they regulate the cell cycle and arrest the cell in the S phase. In the cell, the ribonucleoproteins—proteins with homology to ribosomal protein S12 and chaperonin 10—play a similar role in cell cycle regulation.

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