Identification and characterization of a 66–68-kDa protein as a methotrexate-binding protein in murine leukemia L1210 cells

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Abstract We previously observed an unidentified, tyrosine-phosphorylated, membrane-associated, 66–68-kDa protein which was present in the L1210 murine leukemia cells but not present, at least in the tyrosine-phosphorylated form, in cisplatin–methotrexate (CDDP–MTX) cross-resistant L1210/DDP cells. We purified and characterized this 66–68-kDa protein by affinity chromatography purification using its two identified properties, tyrosine phosphorylation and MTX-binding, and yielded a single band of 66–68 kDa. The purified protein was subjected to trypsin digestion and the isolated peptide

fragments were sequenced and yielded two partial peptide sequences: VEIANDQ and VTNAVVTVPAYFNDSQRQA. The two peptide sequences were used to search for the mouse genome at the national center for biotechnology information (NCBI) database for Open Reading Frame Sequence (ORFs) containing these peptides using the TBLASTN function. A single gene was identified containing both sequences, the *HSPA8* gene, which codes for the heat shock family protein, HSC70. We further demonstrated that HSC70 is a MTX-binding protein using a binding assay with MTX-agarose beads followed by Western blotting. The HSC70 also existed in various cancer cell lines and showed binding to MTX. Additionally, the HSC70 protein, cloned from the L1210 murine leukemia cells, was expressed and purified from *E. coli* cells using a polyhistidine-tag purification system and it also showed the binding properties with MTX. DnaK, the HSC70 homologue in *E. coli*, also binds to MTX. By using the purified truncated HSC70 domains, we identified the adenosine triphosphatase (ATPase) domain of HSC70 that can bind to MTX. Thus, we have tentatively characterized a new, novel property of HSC70 as a MTX-binding protein.

Keywords Leukemia · HSC70 · Drug resistance · Methotrexate · Methotrexate-binding protein

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Introduction

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells and the second leading cause of death in the United States since 1970 (Marker 1968; Siegel et al. 2011). Leukemia, a cancer of the blood or bone marrow, is characterized by abnormal proliferation of blood cells, especially leukocytes (Campana and Behm 2000). Leukemia is the most common cancer in children and young adults (<40 years old) and has been ranked in the top

ten leading cancer types in both males and females in the United States since 2000 (Siegel et al. 2011). There were an estimated 43,050 new cases and 21,840 deaths from leukemia in the United States in 2010 (Siegel et al. 2011). Leukemia can be divided into four major types based on the progression time and cell types: acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), and chronic myelogenous leukemia (CML) (Foon and Todd 1986).

The dramatic improvement in leukemia treatment that began late in the twentieth century is mainly the result of chemotherapy. However, a major problem in the treatment of leukemia is the development of resistance to chemotherapeutic drugs. Drug resistance is a phenomenon in which cancer cells fail to respond to chemotherapy agents (Gottesman 2002). Cisplatin (CDDP) belongs to the family of platinum coordination complexes which enter cells by diffusion. It cross-links cellular DNA, especially N⁶ of guanine (Zwelling and Kohn 1979). The mechanisms of CDDP resistance include, but are not limited to, reduced intracellular CDDP accumulation, enhanced levels of glutathione which binds and inactivates CDDP, and increased rates of DNA replication (Siddik 2003; Zwelling and Kohn 1979). CDDP is one of the most commonly used chemotherapeutic drugs in the treatment of various cancers clinically. Methotrexate (MTX) is a folic acid analog. Folic acid is an essential factor for the synthesis of precursors of DNA and RNA. Dihydrofolate reductase (DHFR) is the enzyme that catalyzes the conversion of dihydrofolate into tetrahydrofolate, which is necessary in DNA and RNA syntheses. MTX has a higher affinity for DHFR than folic acid, thus MTX can inhibit cell growth by binding with DHFR (Jolivet et al. 1983). MTX is used for the treatment of various cancers including leukemia and other diseases such as psoriasis and rheumatoid arthritis. Cross-resistance is a significant problem in cancer therapy with multiple drugs. CDDP-resistant cells can be cross-resistant with many other chemotherapeutic agents including MTX, 5-fluorouracil, melphalan, and 6-mercaptopurine (Hamilton et al. 1985; Newman et al. 1988; Shen et al. 1998). However, the exact mechanisms of CDDP cross-resistance with MTX have not been completely elucidated.

Wroblewski et al. (1996) found that the mouse L1210 cell line, lymphocytic leukemia cells, selected for CDDP resistance (L1210/DDP cells) showed more than 25,000-fold resistance to MTX compared to parental cells (L1210/0 cells). In order to investigate the mechanism leading to MTX resistance in the L1210 cells, Bhushan et al. (1996) have excluded all the well-known MTX-resistant mechanisms including DHFR gene amplification, increased DHFR activity, change in folylpolyglutamate synthetase activity, and decreased MTX binding to its target enzyme DHFR. Thus, there should be some unidentified mechanism leading to the MTX resistance.

Mouse L1210 cells contain a transport system for accumulation of MTX by integral (48 kDa), cytosolic (38 kDa), or peripheral proteins which is involved in transporting substrates of DHFR (Price and Freisheim 1987). MTX could not be transported into the resistant L1210/DDP cells due to the abnormal functions of certain MTX transports (Bhushan et al. 1999). In parental L1210/0 cells, the 66–68-kDa, 48-kDa, 38-kDa, and 21-kDa bands were detected by photo-affinity probes to determine the binding of MTX to proteins (Bhushan et al. 1996). However, all the proteins were detected except for the 66–68-kDa protein in the L1210/DDP-resistant cells. Since there was no association of the affinity label with any other proteins in L1210/DDP cells, it was confirmed that MTX resistance in L1210/DDP cells was not due to DHFR alteration and suggested it may be due to lack of MTX transport (Wroblewski et al. 1996). A significant difference in protein tyrosine phosphorylation status between sensitive and resistant cells (Bhushan et al. 1996) was further demonstrated. The 66–68-kDa protein was not present in the tyrosine-phosphorylated form in L1210/DDP cells, particularly in the membrane fraction (Bhushan et al. 1999). This suggests the 66–68-kDa protein may act as a membrane transporter and inappropriate function of the protein may lead to improper transport of MTX to DHFR in resistant cells. Additionally, genistein, a tyrosine kinase inhibitor, was used to determine the effect of tyrosine phosphorylation in L1210 cells. Increased resistance to MTX, decreased tyrosine phosphorylation of the 66–68-kDa protein, and decreased MTX uptake were observed upon genistein treatment on L1210/0 cells (Xuan et al. 1998). Thus, previous studies suggested a potential relationship among MTX transport, MTX resistance, and state of tyrosine phosphorylation of the unidentified 66–68-kDa protein.

In this study, we purified the 66–68-kDa protein from the membrane fraction of the parental L1210/0 cells with immunoaffinity chromatography based on its properties, MTX association, and tyrosine phosphorylation. In addition, we characterized the 66–68-kDa protein as HSC70 and demonstrated the interaction between HSC70 and MTX.

Materials and methods

Cell culture

Parental L1210/0 cells were purchased from ATCC (Manassas, VA, USA) and CDDP-resistant L1210/DDP cells were established from the parental cells by chronic treatment with 5 μ M of CDDP over a period of 6 months until the resistance was maintained without exogenous CDDP. Both sensitive (L1210/0) and resistant (L1210/DDP) mouse lymphocytic leukemia cells were cultured at 37 °C, 5 % CO₂, in McCoy's 5A medium (Atlanta Biologicals, GA, USA) containing L-

glutamine, and 2.2 g/l sodium bicarbonate (Fischer, NJ, USA). The medium contains 5 % donor horse serum (Atlanta Biologicals, GA, USA) for L1210/0 cells and 5 % fetal bovine serum (Atlanta Biologicals, GA, USA) for L1210/DDP cells.

Cell lysate preparation

Cells (1×10^7) were collected and centrifuged at 1,000 RPM for 5 min and the supernatants were discarded. The cell pellets were washed twice with ice-cold $1 \times$ PBS and resuspended in 100 μ l $1 \times$ cell lysis buffer (2 % Triton X-100, 20 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 60 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.2 % sodium azide, pH=7.6) containing 200 μ M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μ g/ml aprotinin, and 20 μ g/ml leupeptin. The lysates were sonicated. The suspensions were centrifuged at 8,000 RPM for 5 min, and the supernatants were collected and the protein concentration was determined.

Reagents

Primary anti-HSC70 and anti-DnaK antibody were purchased from Abcam (Cambridge, MA, USA). The secondary goat anti-rat IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Immunoaffinity chromatography

L1210/0 cells (5×10^8) were centrifuged at 450g for 5 min at 4 °C. The cell pellets were washed twice with $1 \times$ PBS by centrifugation at 450g for 5 min, and the supernatant was discarded. The cell pellets were then resuspended in buffer A (20 mM HEPES, 4 mM MgCl₂, 1 mM PMSF, 0.02 mg/ml leupeptin, and 0.4 mM sodium orthovanadate) followed by homogenization with a Dounce homogenizer. After the cells were lysed, the homogenates were centrifuged at 3,000g for 10 min and then the supernatants were transferred into ultracentrifuge tubes and centrifuged at 100,000g for 1 h. The supernatants were discarded and the pellets were suspended in 0.4 % saponin in buffer A. The mixture was centrifuged at 100,000g for 1 h and the supernatant was loaded onto an equilibrated MTX agarose column (Sigma-Aldrich, St. Louis, MO, USA). The sample was eluted with 1 M of NaCl and dialysed, and then loaded onto an equilibrated PY20 antibody agarose column (Biomol, Plymouth Meeting, PA, USA). The column was extensively washed with wash buffer (50 mM Tris-HCl, 0.2 % Triton X-100, 0.2 mM Na₃VO₄, 1 mM MoO₄, and 0.25 mM PMSF, pH 7.7). The tyrosine-phosphorylated proteins were eluted with 20 mM of phosphotyrosine solution. Silver staining analysis was performed to confirm the presence of proteins in the eluted samples. The fractions were separated by sodium dodecyl sulfate

polyacrylamide gel electrophoresis (SDS-PAGE) and the band of 66–68 kDa was excised and sent to the University of Texas Health Science Center at San Antonio for amino acid sequence analysis by mass spectrometry.

Bioinformatics analysis

The partial peptide sequences of the protein were used to search the mouse protein database using protein BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Proteins&PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGETYPE=BlastSearch&SHOWDEFAULTS=on). The sequences were entered for query sequence, database chosen was protein data bank protein, organism chosen was mouse, and the algorithm chosen was blastp (protein–protein BLAST). Next, the amino acid sequences of the protein were again used to search the mouse genome at the NCBI database (<http://www.ncbi.nlm.nih.gov/>) for ORFs contacting the amino acid sequences using the TBLASTN tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Translations&PROGRAM=tblastn&BLAST_PROGRAMS=blastn&PAETYPE=BlastSearch&SHOWDEFAULTS=on). The sequences were entered for query sequence, database chosen was reference genomic sequences, and organism chosen was mouse.

Methotrexate binding assay

One hundred microliters of MTX agarose beads (Sigma-Aldrich, St. Louis, MO, USA) were used for each sample. To prepare the MTX agarose beads for a binding assay, the beads were centrifuged at 300 RPM for 3 min and the supernatants were discarded. The beads were washed subsequently with 1 ml ice-cold $1 \times$ PBS and $1 \times$ cell lysis buffer twice. Eighty microliters of the cell lysates, 20 mg of purified HSC70 protein, or HSC70 fragments in 80 μ l volume was added and mixed with the MTX agarose beads and mixed on a rocker overnight at 4 °C allowing them to mix and interact thoroughly. The next day, the beads and protein mixtures were centrifuged at 300 RPM for 3 min and the supernatant were discarded. The beads were subsequently washed three times with $1 \times$ cell lysis buffer. Eighty microliters of $1 \times$ SDS sample buffers was added and mixed with the beads, and the mixtures were incubated in a 95 °C water bath for 5 min. The samples were subjected to Western blotting assay.

Western blotting

MTX-binding proteins, different cell lysates, or purified samples were loaded on a 12 % SDS-polyacrylamide gel, separated by electrophoresis, and subsequently, transferred to a PVDF membrane. The membranes were blocked with 5 % milk in $1 \times$ tris buffered saline (TBS) containing 0.05 % (v/v) Tween for

4 h. The membranes were washed seven times with 1× TBS and 1× tris buffered saline with tween (TBST) alternatively. The membranes were incubated with primary antibody (1:1,000 dilution) at 4 °C overnight, followed by incubation with secondary antibody (1:2,500 dilution) at room temperature for 1 h. Pierce SuperSignal Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) was used and image was captured using an X-ray scientific imaging film.

Silver staining analysis

Twenty-five microliters of the protein sample mixed with 5 µl of 6× nondenaturing sample buffer was loaded on a 12 % SDS-polyacrylamide gel and was separated with electrophoresis. The gel was then carefully collected and placed in a plastic container. Fifty milliliters of formaldehyde fixing solution (Fisher Scientific, Pittsburgh, PA, USA) was added to the gel and the gel was agitated gently on an orbital shaker for 10 min. The fixing solution was poured, the gel was washed twice for 5 min each with water, and the water was removed. Next, the gel was soaked in 50 ml 0.2 g/l Na₂S₂O₃ solution (Sigma-Aldrich, St. Louis, MO, USA) with slow agitation for 1 min. Then, the Na₂S₂O₃ solution was poured and the gel was washed twice with water for 20 s. Next, the water was poured, and the gel was soaked in 50 ml of 0.1 % silver nitrate (Sigma-Aldrich, St. Louis, MO, USA) with slow agitation for 10 min. After removing the silver nitrate solution, the gel was washed first with water and then with 25 ml of thiosulfate (Sigma-Aldrich, St. Louis, MO, USA) developing solution. Finally, the gel was soaked in 50 ml of fresh thiosulfate developing solution and agitated slowly until band intensities are adequate (around 1–5 min). After the bands clearly showed up, 5 ml of 2.3 M citric acid (Sigma-Aldrich, St. Louis, MO, USA) per 100 ml thiosulfate developing solution was added to the gel to stop the reaction.

HSC70 purification

The cDNA from L1210 mouse leukemia cells was isolated and purified with the QIAGEN PureGene Kit following the manufacture's protocol (Valencia, CA, USA). A six-histidine tag was attached to either amino- or carboxyl-terminal of HSC70 by polymerase chain reaction (PCR). The forward primer to insert the six-histidine tag into an amino terminal was 5'-TGAGAGGAATAATAAATGCATCATCACCATCACCATCTTAAGGGACCTGCA GTTGGC-3', and the reverse primer was 5'-TTAATCACCTCTTCAATGGTGGGGCC-3'. The forward primer to insert the six-histidine tag into carboxyl-terminal was 5'-TGAGAGGAATAATAAATGTCTAAGGGACCTGCAGTTGGC-3', and the reverse primer was 5'-TTAATGGTGTATGGTGTATGATGATCCACCTC

TTCAATGGTGGGGCC-3'. The PCR reaction is performed with denaturation at 95 °C for 10 min, annealing at 53 °C for 1 min, and extension at 72 °C for 4 min, total 35 cycles. The PCR product was inserted into pBAD-TOPO vector following the manufacture's protocol (Invitrogen, Carlsbad, CA, USA). The plasmid was then transformed into *E. coli* cells by heat shock following the manufacture's protocol. Positive clones were picked up and verified and were then cultured in 125 ml of luria broth (LB) media in conical flasks at 37 °C for 2 days, and 0.02 % of arabinose (Alfa Aesar, Ward Hill, MA, USA) was added to the cells to induce the protein expression for 24 h. The *E. coli* pellets were collected by centrifugation at 10,000 RPM for 30 min, and the cells were lyzed in 6 ml of 100 mM HEPES buffer with 10 mM imidazole and 1 mM PMSF by bead beating for 5 min. Three hundred microliters of the nickel-chelating ProBond resin (Invitrogen, Carlsbad, CA, USA) was washed with 1 ml of water, followed by washing with 1 ml of washing buffer (100 mM HEPES, 10 mM imidazole, and 500 mM NaCl). Nine hundred microliters of cell lysate was added to the resin and was put on a horizontal shaker for 1 h at 4 °C allowing the full interaction of the cell lysates with the beads. The resin was then centrifuged at 1,000 RPM for 30 s, and the supernatant was discarded. The resin was then washed three times with 1 ml washing buffer. Finally, 900 µl of elution buffer (100 mM HEPES and 500 mM imidazole) was added to the resin and put on the horizontal shaker for 1 h at 4 °C. The purified protein was in the elution solution and was analyzed by Western blotting and silver staining assay.

APTase domain of HSC70 binds to MTX

Twenty micrograms of different purified truncated HSC70 domain in 80 ml volume were used for binding with the MTX agarose beads for identification of the domain of HSC70. The purified truncated murine HSC70 was generously given by Dr. Eileen M. Lafer at University of Texas Health and Science Center at San Antonio. The purified adenosine triphosphatase (ATPase) domain about 44 kDa and the remaining two other domains (substrate-binding domain and carboxyl domain) about 28 kDa were used to bind with the MTX agarose beads followed by analysis with Western blotting. The purified HSC70 from *E. coli* was used as a positive control.

Results

Purification and characterization of the 66–68-kDa protein by immunoaffinity chromatography

Based on the properties of the unidentified 66–68-kDa protein (tyrosine phosphorylation, affinity for MTX, and membrane association), membrane fractions of L1210/0 cells

were isolated and treated with 0.04 % saponin. The membrane fractions from the L1210/0 cells were subjected to immunoaffinity chromatography, first with MTX agarose column followed by the PY20 antibody agarose column. After the membrane-bound proteins were subjected to the MTX agarose column, a major 66–68-kDa band and several other bands were observed (Fig. 1a, lane 1). The eluted samples from the MTX agarose column were further purified with the PY20 antibody agarose column and the presence of the tyrosine-phosphorylated proteins were confirmed by silver staining assay. Only one 66–68-kDa band was presented indicating the purification of the protein from the membrane fraction (Fig. 1a, lanes 2, 3, and 4). Additionally, the 66–68-kDa protein was not detected in the washing solution but only presented in the elution buffer after purification through the PY20 antibody agarose column (Fig. 1b). The result suggested that the 66–68-kDa protein bound to the PY20 antibody agarose beads in the column. Next, the elution sample was separated on a SDS-PAGE and the appropriate 66–68 kDa was excised. The excised band was isolated and sent to the University of

Texas Health Science Center at San Antonio for amino acid sequences analysis. Subsequent trypsin digestion and sequence analysis by mass spectrometry of fragments yielded two partial peptide sequences: (1) VEIANDQ and (2)TVTNAVVTVPAYFNDSQRQA.

Identification of the 66–68-kDa protein as the HSC70 protein using bioinformatics tools

In order to identify the 66–68-kDa protein, the two peptide sequences (VEIANDQ and VTNAVVTVPAYFNDSQRQA) obtained from the peptide fragments of this protein were used to search the mouse genome at the NCBI database (<http://www.ncbi.nlm.nih.gov/>) for ORFs containing these peptides using the TBLASTN function. After the searching, we found that only a single gene was identified containing both of the two sequences. It is the *HSPA8* gene, which codes for the heat shock cognate protein 70, HSC70. Table 1 showed the amino acids sequence of the mouse HSC70 protein with the two identical peptide sequences of the 66–68-kDa protein obtained from the NCBI database. The bioinformatics result indicated the unidentified 66–68-kDa protein in L1210/0 cells is the HSC70 protein.

HSC70 is a MTX-binding protein in both L1210/0 and L1210/DDP cells

We identified the 66–68-kDa protein as the HSC70 protein belonging to the heat shock protein 70 family. In order to investigate the relationship of this protein and MTX, we performed the Western blotting assay with total cell lysate. We found that HSC70 and DHFR (the MTX target enzyme) were present in both L1210/0 and L1210/DDP cells (Fig. 2a). Next, we determined the putative relationship between HSC70 and MTX. MTX-binding assay was performed to study the binding properties of the HSC70 protein with MTX. Eighty microliters of the cell lysates was used to bind with MTX agarose beads. The MTX-bound proteins were then separated on a SDS-PAGE and Western blotting analysis confirmed the presence of HSC70. HSC70 from both L1210/0 and L1210/DDP cell lysates showed the binding properties with MTX agarose beads (Fig. 2b, lanes 1 and 3). MTX can compete with folate acid for binding to DHFR; thus, DHFR was used as a positive control for binding to MTX. DHFR could bind to the MTX agarose beads in both L1210 cell lines. As a negative control, beta-actin did not bind to MTX agarose beads (Fig. 2b). We also used the agarose beads conjugated with glycine as a negative control because HSC70, DHFR, and beta-actin could not bind with the glycine agarose beads (Fig. 2b, lanes 2 and 4). The study showed that HSC70 is a putative MTX-binding protein in L1210 cells.

Fig. 1 Purification of the 66–68-kDa protein by immunoaffinity chromatography with MTX agarose column (**a**) and followed by PY20 antibody agarose column (**b**). The band of 66–68 kDa was the major band presented in the elution sample after purification through the MTX agarose column (**a**: lane 1). The 66–68 kDa was presented in a single band in the elution sample after further purification through the PY20 antibody agarose column (**a**: lanes 2, 3, and 4 and **b**: lane 2). The band of 66–68 kDa was not presented in the washing solution after purification through the PY20 agarose column (**b** lane 1)

Table 1 The amino acid sequence of mouse HSC70 protein obtained from the NCBI database

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MSKGPVAVGIDLGTTYSVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDA
AKNQVAMNPTNTVFDAKRLIGRRFDDAVVQSDMKHWPFMVNDAGRPKVQV
EYKGETKSFYPEEVSSMVLTKMKEIAEAYLGKTVTNAVVTVPAYFNDSQRQA
TKDAGTIAGLNVLRIINEPTAAAIAYGLDKKVGAEARNVLIFDLGGGTDFVSILTIE
DGIFEVKSTAGDTHLGGEDFDNRMVNHFAIEFKRKHKKDISENKRAVRRRLTA
CERAKRTLSSSTQASIEIDSLYEGIDFYTSITRARFEELNADLFRGTLDPVEKALR
DAKLDKSQIHDIVLVGGSTRIPKIQKLLQDFFNGKELNKSINPDEAVAYGAHVQ
AAILSGDKSENVQDLLLDVTPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTY
SDNQPGVLIQVYEGERAMTKDNNLLGKFELTGIPPAPRGVVPQIEVTFDIDANGIL
NVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAKEYKAEDEKQRDKVSSK
NSLESYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIISWLDKNQTAEKEEFE
HQQKELEKVCNPIITKLYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD

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The mouse HSC70 protein is composed of 646 amino acids. The sequences of the two peptide fragments from the 66–68-kDa protein are VEIIANDQ and VTNAVVTVPAYFNDSQRQA. The mouse HSC70 protein is the only mouse protein comprising the identical two peptide sequences from the 66–68-kDa protein which are highlighted in the table indicating the 66–68-kDa protein is HSC70

HSC70 binds with MTX in various cancer cell lines

HSC70 is a putative MTX-binding protein in L1210 cells, and we further explored the roles of HSC70 and

Fig. 2 MTX-binding assay for HSC70 with total cell lysates (**a**) and MTX or glycine agarose beads (**b**) in L1210/0 and L1210/DDP cells. **a** Lane 1: L1210/0 (sensitive cells) and lane 2: L1210/DDP (resistant cells). **b** Cell lysates were prepared, mixed, and incubated with 100 μ l of MTX agarose beads (lanes 1 and 3), or 100 μ l of glycine agarose beads (lanes 2 and 4), and the MTX-bound proteins were separated on a SDS-PAGE followed by detecting the presence of HSC70 with Western blotting assay. Lane 1 L1210/0 (sensitive cells), lane 2 L1210/0 (sensitive cells) with glycine agarose beads, lane 3 L1210/DDP (resistant cells), lane 4 L1210/DDP (resistant cells) with glycine agarose beads. Beta-actin was used as negative controls. DHFR was used as positive controls. The blots shown are representative of two independent experiments

MTX in other cancer cells. We want to clarify whether the putative binding property of HSC70 with MTX is limited to the L1210 cells, or if the property was also presented in other cancer cells. We tested the binding characteristics of HSC70 with MTX agarose beads in various cancer cell lines including human astrocytoma U87 cells, human neuroblastoma SK-N-SH cells, murine multiple myeloma 7TD1 cells, human pancreatic cancer AsPC cells, human pancreatic cancer PANC1 cells, and human pancreatic cancer PANC10.05 cells. Our results showed that HSC70 was present in the total cell lysate (Fig. 3a) and it bound to the MTX agarose beads (Fig. 3c), while HSC70 could not bind with the glycine agarose beads as the negative controls (Fig. 3b) in all the tested cancer cell lines.

Fig. 3 MTX-binding assay for HSC70 with total cell lysate (**a**), glycine agarose beads (**b**), and MTX agarose beads (**c**) in various cancer cell lines. The tested cell lines include U87 (human astrocytoma cells), SK-N-SH (human neuroblastoma cells), 7TD1 (murine multiple myeloma cells), AsPC (human pancreatic cancer cells), PANC1 (human pancreatic cancer cells), and PANC10.05 (human pancreatic cancer cells). The blots shown are representative of two independent experiments

Expression and purification of HSC70 from *E. coli* cells and the binding property with MTX

We investigated the roles of HSC70 with MTX in prokaryotic cells and cloned the *HSPa8* gene from L1210/0 cells and induced the expression of the HSC70 protein in *E. coli* followed by purification of the HSC70 protein with a polyhistidine-containing protein purification system. Western blotting and silver staining assay were used to detect the presence of proteins. We optimized the concentration of arabinose to induce the expression of the polyhistidine tagged HSC70 in *E. coli*. HSC70 expression was detected in the *E. coli* cells which were induced by 0.02 % arabinose, but not in the uninduced samples (Fig. 4a, lane 1 and 2). Next, we purified the HSC70 based on the affinity of

Fig. 4 Detection of purified HSC70 protein from the *E. coli* lysates with Western blotting (**a**), silver staining assay (**b**), and MTX-binding assay for the purified HSC70 (**c**). The *HSPa8* gene from L1210/0 cells was cloned into the pBAD plasmid and the expression of HSC70 was induced by 0.02 % arabinose in *E. coli* cells followed by purification of the HSC70 with affinity chromatography. The HSC70 purification products were detected by Western blotting and silver staining assay. **a** Lane 1 uninduced sample, lane 2 crude lysate, lane 3 first washing solution, lane 4 second washing solution, lane 5 elution. HSC70 was not detected in the sample without the induction by arabinose (lane 1), only little HSC70 was detected in the washing solutions (lanes 3 and 4), and significant HSC70 was detected in crude lysates and elution with arabinose induction and elution solution (lanes 2 and 5). **b** Lane 1 crude lysate, lane 2 first washing solution, lane 3 second washing solution, lane 4 elution. Multiple bands were detected in crude lysates and washing solutions (lanes 1, 2, and 3) and only a single 70 kDa band was detected in the elution solution (lane 4). **c** Lane 1 *E. coli* crude lysates, lane 2 *E. coli* crude lysates binding with MTX agarose beads, lane 3 purified HSC70, lane 4 purified HSC70 binding with MTX agarose beads. The HSC70 could not be detected in normal *E. coli* crude lysate samples (lanes 1 and 2) but the purified HSC70 was detected in the arabinose-induced *E. coli* lysates as well as after binding with MTX agarose beads (lanes 3 and 4). The blots shown are representative of two independent experiments

the polyhistidine-tag with nickel resin; only a low level of HSC70 was detected in the first washing solution and was not detected in the second washing solution. HSC70 was presented in the elution solution and suggested the protein was purified in the elution solution from the crude *E. coli* lysates (Fig. 4a, lanes 3, 4, and 5). Moreover, the silver staining assay showed only one 70-kDa band was presented in the elution sample, suggesting the HSC70 protein was purified (Fig. 4b). In order to further explore the relationship between MTX and HSC70, both the crude lysate from the *E. coli* cells and the purified HSC70 were used for MTX-binding assay. We found that the purified HSC70 from *E. coli* cells also showed binding properties with the MTX agarose beads (Fig. 4c, lanes 3 and 4).

DnaK can bind to MTX

DnaK is the HSC70 homologue in *E. coli* and performs similar functions as HSC70 in mammalian cells. DnaK in *E. coli* and HSC70 in mammalian cells is a member of the HSP70 family that shows high similarity in sequences and structures, as well as some comparable functional properties such as interactions with some other proteins and cooperation with co-chaperones. We compared the amino acid sequence between mouse HSC70 and DnaK with a ClustalW sequence alignment of the primary sequences of HSC70 and DnaK in FASTA format and the data showed more than 50 % of the sequence homology between them (Table 2). Next, we performed the MTX-binding assay using the *E. coli* lysates, followed by detection of the presence of DnaK among the proteins binding with MTX agarose beads by Western blotting. The results show that DnaK antibody could not recognize HSC70 in L1210/0 cells (Fig. 5, lanes 1 and 2) and the DnaK in *E. coli* has the binding properties with MTX (Fig. 5, lane 4) but it could not bind with the glycine agarose beads (negative control, Fig. 5, lane 5).

ATPase domain of HSC70 binds to MTX

To identify which domain of HSC70 is involved in binding with MTX, we tested different purified truncated HSC70 domains with the MTX agarose bead binding assay. The purified ATPase domain about 44 kDa and the remaining other two domains (substrate-binding domain and carboxyl domain) about 28 kDa were used to bind with the MTX agarose beads, while the purified HSC70 from *E. coli* was used as a positive control (Fig. 6, lane 2). After binding with MTX agarose beads followed by Western blotting, we found that the purified ATPase domain can bind with MTX (Fig. 6, lane 4); however, the remaining two domains of HSC70 including the substrate-binding domain and carboxyl-terminal domain could not bind with MTX agarose beads (Fig. 6, lane 6). These results showed that MTX is able to bind to the ATPase domain of HSC70.

Fig. 6 Identification of the domain of HSC70 binding with MTX. MTX-binding assay was performed using the purified and truncated murine HSC70 proteins and Western blotting was used to detect the HSC70. *Lane 1* purified HSC70 protein from *E. coli* (70 kDa), *lane 2* purified HSC70 binding with MTX agarose beads, *lane 3* purified HSC70 binding with glycine agarose beads, *lane 4* purified ATPase domain of murine HSC70 (44 kDa), *lane 5* purified ATPase domain of murine HSC70 binding with MTX agarose beads, *lane 6* purified ATPase domain of murine HSC70 binding with glycine agarose, *lane 7* purified substrate binding domain and carboxyl domain of murine HSC70 (28 kDa), *lane 8* purified substrate binding domain and carboxyl domain of murine HSC70 binding with MTX agarose beads, *lane 9* purified substrate binding domain and carboxyl domain of murine HSC70 binding with glycine agarose. Western blotting analysis showed that both purified HSC70 from *E. coli* and purified ATPase domain of murine HSC70 protein were detected in the samples binding with MTX agarose beads (*lanes 2* and *5*) but not the purified substrate binding domain and carboxyl domain of murine HSC70 protein could not be detected in the samples binding with MTX agarose beads (*lane 8*). All the purified proteins and truncated protein domains could not be detected in the sample binding with glycine agarose beads (*lanes 3, 6, and 9*). The blots shown are representative of two independent experiments

route for the uptake of MTX (Fan et al. 1992). RFC is the primary transporter of folates and antifolate chemotherapeutic drugs such as MTX, pemetrexate, and raltitrexed in mammalian cells and tissues (Kaufman et al. 2003).

Aberrant functions of MTX transports can be the obstacle of successful transportation of MTX into cancer cells and may further lead to MTX resistance in cancer therapy. Previous studies by Bhushan et al. (1996) showed that a tyrosine-phosphorylated 66–68-kDa protein was present in the L1210/0 cells but absent in the L1210/DDP cells. In L1210/0 cells, the 66–68-kDa, 48-kDa, 38-kDa, and 21-kDa bands were detected by photoaffinity probe to determine the binding of MTX to DHFR. However, the 66–68-kDa protein was not detected in the resistant L1210/DDP cells. It has been demonstrated that MTX resistance was not due to DHFR alteration. Further studies suggested that MTX resistance in L1210/DDP cells may be due to lack of MTX transport (Wroblewski et al. 1996). The membrane-bound 66–68-kDa protein may act as a membrane transport and inappropriate function of the protein may lead to improper transport of MTX to DHFR in resistant cells.

In our present study, based on the two properties of the unidentified 66–68-kDa membrane-associated protein, tyrosine phosphorylation, and affinity to MTX, we isolated and

purified the protein by immunoaffinity chromatography. After the membrane-bound protein was first purified with the MTX agarose column, some other bands were also presented in the elution buffer in addition to the major 66–68-kDa band suggesting the presence of other MTX-binding proteins in the membrane fraction (Fig. 1, lane 1). However, only the 66–68-kDa band was present after further purification with the PY20 antibody agarose column. The results suggested that the 66–68-kDa protein from the L1210/0 cells was tyrosine phosphorylated (Fig. 1, lanes 2, 3, and 4). The peptide fragments from the protein were sequenced and yielded two peptide sequences. Furthermore, we identified the 66–68-kDa protein as HSC70 using bioinformatics tools. HSC70 is a constitutively expressed heat shock protein and belongs to the HSP70 family, sharing 85 % amino acid similarity with HSP70 (Ali et al. 2003; Boone and Vijayan 2002).

The 66–68-kDa protein is a membrane-associated protein in L1210 cells (Bhushan et al. 1999). Although HSC70 is a major cytosolic molecular chaperone, it is also located in other cellular locations such as the cellular membrane and nucleus (Dworniczak and Mirault 1987; Wubbolts et al. 2003). HSC70 can directly interact with a lipid bilayer in the cellular membrane and form ion conductance channels, allowing ion flow through in the cellular membrane (Arispe and De Maio 2000). In eukaryotic cells, under normal growth conditions, cytoplasmic HSC70 can move in and out of the nucleus and between nucleus and cytoplasm (Kodiha et al. 2005). Thus, HSC70 may be present in the membrane fraction of L1210 cells and participate in the regulation of MTX transport in L1210 cells.

As a molecular chaperone, HSC70 can interact with a variety of cellular molecules and chemicals. HSC70 needs some co-chaperones including BAG-1, Hip, and Hop to perform its full functions (Gebauer et al. 1998; Höhfeld et al. 1995; Liu et al. 2012). HSC70 has been shown to interact with many other proteins and chemicals as well. For instance, a 16-kDa protein (p16), a member of the Nm23/nucleoside diphosphate (NDP) kinase family, had been identified as a new regulatory protein for HSC70 (Leung and Hightower 1997). Receptor-associating protein 46 which regulates the transaction function of steroid can interact with the ATPase domain of HSC70 and is a regulator of HSC70 (Schneikert et al. 2000). The thrombospondin type 1 repeat proteins interact with the substrate-binding domain of HSC70 in mouse macrophage cells (Ihara et al. 2010). The arylhydrocarbon receptor-interacting protein can bind with the carboxyl-terminal amino acid residues (EEVD) (Yano et al. 2003). Small glutamine-rich TPR-containing protein can interact with the carboxyl-terminal of HSC70 (Tobaben et al. 2001). HSC70 can also interact with some drugs and natural compounds such as deoxyspergualin (DSG), an immunosuppressive drug. DSG binds tightly with the extreme

carboxyl-terminal four-amino acid sequence EEVD in HSC70 (Nadler et al. 1998). HSC70 was expressed much higher in some cancer cell lines, such as lung small cell carcinoma and breast adenocarcinoma, compared to normal cell lines (Maeda et al. 2000). However, no study has been reported regarding the relationship between MTX and HSC70. This led us to search the pathway studio analysis to determine if there was any correlation between MTX and HSC70. So far, no direct interaction has been found (Nikitin et al. 2003). In order to elucidate the potential relationship between HSC70 and MTX, we performed the MTX-binding assay using MTX agarose beads and found that HSC70 may be a putative MTX-binding protein in L1210 cells. In order to confirm our finding, beta-actin was used as the negative control because beta-actin could not bind to MTX agarose beads. We also used the glycine agarose as a negative control because MTX could not bind with the glycine agarose beads (Fig. 2). In addition, we also used the cyanogen bromide activated matrices, and HSC70 could not bind to the negative beads (data not shown). We further used different amounts of MTX agarose beads (from 50 to 200 μ l) to bind with a fixed amount of L1210 cell lysates and we observed that higher amounts of HSC70 lysates was detected with increased volume of MTX agarose beads in L1210 cell. The data suggest that MTX can bind with MTX in L1210 cells (data not shown). Furthermore, we used a variety of cancer cell lines including murine multiple myeloma, human neuroblastoma, human astrocytoma, and human pancreatic cancer cells to investigate whether the HSC70 can bind to MTX in these cell lines. The MTX-binding assay results showed that HSC70 had the binding properties with MTX in all the tested cancer cells (Fig. 3). These studies suggest that binding of HSC70 to MTX may be a general role in most cancer cells. In order to further explore the properties of HSC70, we cloned the *HSPA8* gene and expressed and purified HSC70 in *E. coli*. We found the purified HSC70 in *E. coli* cells can also bind to MTX (Fig. 4c). Thus, the binding characteristics of HSC70 to MTX may be a general role, not only limited to cancer cell lines. DnaK is a member of the HSP70 molecular chaperone family in *E. coli*. In bacteria, DnaK performs similar functions as HSC70 in mammalian cells such as the ATPase activity. DnaJ, also known as HSP40 in eukaryotic cells, works as the co-chaperone of DnaK for the performance of the full function of DnaK. We did a ClustalW sequence alignment of the primary sequences of HSC70 and DnaK in FASTA format showing more than 50 % of the sequence homology between them (Table 2). MTX-binding assay with MTX agarose beads suggested that DnaK also has the binding properties with MTX (Fig. 5).

HSC70 is a well-characterized ATP-binding chaperone and has intrinsic ATPase activity (Jakob et al. 1996; Liu et al. 2012). HSC70-hydrolyzing ATP can initiate the

conformational change of HSC70 and further cause substrate binding by HSC70 (Sullivan and Pipas 2002). Thus, the activation of HSC70 by ATP binding to the ATPase domain may cause the binding to MTX. The murine HSC70 comprises 646 amino acids and the basic structure of HSC70 includes three parts: a 44-kDa amino terminal ATPase domain (residues 1–384), a 18-kDa peptide (substrate)-binding domain (residues 385–543), and a 10-kDa carboxyl-terminal domain (residues 544–646) which is also designated as the variable or “lid” domain (Tsukahara et al. 2000). The carboxyl-terminal amino acid sequence Glu-Glu-Val-Asp (EEVD motif), which is absolutely conserved in all eukaryotic HSC70 and HSP70 family members, is essential for association with some co-chaperones (Mosser et al. 2000). In the study, we used the purified truncated HSC70 proteins to identify which domain of the HSC70 protein is involved in MTX binding and we found that the ATPase domain of HSC70 can bind to MTX agarose beads, while the substrate-binding domain and carboxyl-terminal domain of HSC70 are not involved in MTX binding (Fig. 6). The results suggest that ATP and MTX can interact with the same domain of HSC70; however, the potential relationship between ATP and MTX on HSC70 is not elucidated and whether the two molecules share the exact binding sites in HSC70 is still unclear. Some other proteins such as receptor-associating protein 46 also interact with the ATPase domain of HSC70, thus studying those ATPase domain-interacting proteins may provide additional information on how HSC70 interacts and regulates other proteins functions, and we can use the information to further explore the possible relationship between different ATPase domain-binding proteins as well.

RFC is the major MTX transporter in L1210 cells (Witt et al. 2004). The structure of hRFC includes 12 mostly hydrophobic transmembrane domains (TMDs) with a large central cytosolic loop composed of 66 amino acids connecting TMDs 6 and 7, and cytosolic-facing amino and carboxyl-terminal (Liu and Matherly 2002; Witt et al. 2004). TMDs 4, 5, 7, 8, 10, and 11 of hRFC forms are structurally and functionally important for folate substrate binding, and they may form a putative channel allowing the binding of substrates (Hou et al. 2005, 2006). Lysine 411 in TMD11 is an important determinant of substrate selectivity and carrier function (Deng et al. 2008; Witt and Matherly 2002). The TMD 6/7 linker region provides the proper partial orientation between the two domains of the carrier for optimal transport function (Liu et al. 2003). Remarkably, HSC70 does not share structural similarities with some well-studied MTX-binding proteins and transporters such as RFC and they differ in molecular weight as well. Based on our studies, HSC70 is a MTX-associated protein which may mediate the transportation of MTX into the cell. As a molecular chaperone, HSC70 may associate and cooperate with

other MTX-interacting proteins participating in the transport of MTX into the cells.

Resistance to chemotherapy agents is a major obstacle to cancer chemotherapy. Wroblewski et al. (1996) reported that mouse leukemia L1210 cells resistant to CDDP exhibited cross-resistance to MTX [12]. Bhushan et al. (1996) have also demonstrated that this resistance is not due to well-established mechanisms. Interestingly, HSC70 has been shown to interact with the nucleotide-binding domain of p-glycoprotein (P-gp) and may participate in the folding of the protein (Loo and Clarke 1995). P-gp is involved in MTX resistance and the interaction between HSC70 and P-gp suggests the possible roles of MTX resistance played by HSC70 (Gottesman et al. 1996).

The other important characteristic of the 66–68-kDa protein is tyrosine phosphorylation. Bhushan et al. (1996) observed the 66–68-kDa protein is tyrosine phosphorylated in sensitive L1210/0 cells but it remains unphosphorylated in the resistant L1210/DDP cells. Bioinformatics analysis indicated several tyrosine sites with high possibility to be potentially phosphorylated in HSC70. KinasePhos analysis showed tyrosines 288, 431, and 525; Motif Scan software showed tyrosines 41 and 288; and NetPhos software showed Y41, 288, and 431 in HSC70 have high potential to be phosphorylated (Blom et al. 1999; Huang et al. 2005; Obenauer et al. 2003). Thus, HSC70 is a tyrosine-phosphorylated protein and tyrosine phosphorylation may play a role in regulating HSC70 functions.

In conclusion, we revealed the possible important role of *HSPA8* gene/HSC70 in the MTX-resistant mechanism in the present study. This is the first report that HSC70 is a putative MTX-binding or MTX-interacting protein and the APTase domain of HSC70 is involved in MTX binding. *HSPA8* gene/HSC70 may be a candidate of biomarker for CDDP–MTX multidrug-resistant leukemia. Furthermore, the study of the molecular mechanism of *HSPA8* gene/HSC70 may help to overcome the multidrug resistance of leukemia. *HSPA8* gene/HSC70 may become a potential novel target for the discovery and development of new chemotherapeutic drugs to treat leukemia and other types of cancer.

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