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Cyclophilin A is upregulated in small cell lung cancer and activates ERK1/2 signal

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Abstract

Cyclophilin A (CypA), a peptidyl–prolyl cis–trans isomerase (PPIase), was originally identified as the intracellular receptor for cyclosporin A (CsA). Recently, correlations of CypA with tumor pathogenesis have been studied. Here, we studied the expression of CypA and its receptor CD147 in several kinds of lung cancer cells as well as a normal lung cell and found that in H446 cell, a kind of small cell lung cancer cell, the expression are the highest. The exogeneous CypA protein can substantially stimulate H446 cell growth in dependence on its PPIase activity. We also showed that CypA protein can stimulate ERK1/2 signal in dose and time dependent manners and almost has no effect to p38 and JNK signals. Elucidation of the precise role of CypA in these pathways may lead to new targeted therapies for small cell lung cancer.

Keywords: Cyclophilin A; Small cell lung carcinoma; ERK1/2; c-Jun NH2-terminal kinase; p38 kinase

Lung cancer is the predominant cause of cancer deaths in industrialized countries. Conventional therapeutic treatments, including surgery, chemotherapy, and radiation therapy, have achieved only limited success. Lung cancer can be divided into four types from histological differences: small-cell carcinoma; squamouscell carcinoma; adenocarcinoma and large-cell carcinoma [1,2].

Cyclophilin A (CypA), a peptidyl–prolyl cis–trans isomerase, was originally identified as the intracellular receptor for cyclosporin A (CsA). In some recent reports, correlations of CypA with tumor pathogenesis have been studied. It is substantially upregulated in various types of cancers and cancer lines such as pancreatic adenocarcinoma, hepatocellular carcinoma, non-small cell lung cancer, several oral cancer cell lines and buccal squamous cell carcinomas [3–6].

Mitogen-activated protein kinases (MAPK) are proline-directed serine/threonine kinases that transmit signals from the cell membrane to the nucleus. In the nucleus, MAPK activate various transcription factors, which promote proliferative and/or inflammatory responses [7–10]. Therefore, these pathways could play a role in malignant transformation or malignant cell growth. The three relatively well-described pathways, which are interconnected, are JNK (c-Jun NH2-terminal kinase), extracellular regulated kinase (ERK), and p38 pathways [11–13]. The ERK1/2 pathway is thought to be stimulated predominantly by growth factors and plays an important role in cell growth and differentiation.

Here we show that CypA and its receptor CD147 are both upregulated in small cell lung cancer cells. The exogeneous CypA protein can stimulate H446 cell growth in dependence on its PPIase activity. CypA protein can

Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase; SDS–PAGE, sodium dodecyl sulfatepolyacrylamidegel electrophoresis; PVDF, polyvinylidene difluoride; RT-PCR, reverse transcription-polymerase chain reaction.

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stimulate ERK1/2 signal in dose and time dependent manners. These observations indicate an important role for CypA in small cell carcinoma tumorigenesis, and suggest its usefulness as a biomarker for small cell lung cancer.

Materials and methods

Plasmids construct. Protein for expression in *Escherichia coli* strain BL21-CodonPlus (DE3), human CypA cDNA (GenBankTM Accession No. NM_021130) was inserted in-frame into pGEX-4T-1 (Amersham Pharmacia Biotech.). CypA-R55A mutant was conducted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Preparation of recombinant proteins. The recombinant glutathione *S*-transferase (GST) tagged proteins were expressed in *E. coli* strain BL21-CodonPlus (DE3) with 0.5 mM IPTG at 25 °C overnight. Cells were collected and lysed in PBS (PH 7.4), supplemented with 0.1% Triton X-100, 1 mM PMSF and 1 mg/ml lysozyme. After sonication on ice for 30 min, the samples were centrifuged and the supernatants were purified using GST beads (Amersham Biosciences). Thrombin (Novagen) was used to cleave recombinant protein to get CypA protein without GST tag.

RNA extraction from cells. Total RNA was extracted from four lung cancer cell lines: H446, H1299, SPC-A1 and A549 cells, as well as normal lung cell Hs888lu using Trizol method. Briefly, 1 ml Trizol reagent was added to 5×10^6 cells and then mixes the suspension with pipettes until all cells debris dissolves. Add 0.2 ml chloroform, mix gently, let stand for 10 min at room temperature followed by centrifugation at 12,000 rpm for 15 min at 4 °C. The upper, clear part was added into a new tube mixed with 0.5 ml isopropanol at room temperature for 10 min and centrifuge at 12,000 rpm for 10 min at 4 °C. 1 ml 75% ethanol wash the pellets, air dry for 10 min. Dissolve pellets in proper amount DEPC treated H_2O.

Real-time RT-PCR. The mRNA levels for CypA, CD147 were analyzed by real-time reverse transcriptase polymerase chain reaction (RT-PCR) using the iCycler system (Bio-Rad, Hercules, CA). The mRNA was reversetranscribed into cDNAs using the iScript cDNA synthesis kit (Bio-Rad). PCR reaction included the following components: 100 nM each primer, diluted cDNA templates and 2× iQ SYBR Green supermix, running for 40 cycles at 95 °C for 20 s and 60 °C for 30 s followed 72 °C for 20 s. Each cDNA sample was run in triplicate. The mRNA level of each sample for each gene was normalized to that of the GAPDH mRNA. The relative mRNA level was presented as unit values of $2^{(Ct\ [GAPDH]\ -\ Ct\ [gene\ of\ interest])}$. The primers of each gene are listed in Table 1.

Cell proliferation assay. H446 cells were seeded in 96-well plate (2×10^3cells/well) and serum-starved for 24 h before adding human CypA protein (0, 0.01, 0.05, 0.1, 0.2, 0.5, 1 nM) or CypA protein incubated with 0.1 μM CsA and CypA-R55A mutanted protein (0, 0.01, 0.1, 1, 5, 10 nM). It is reported that R55A mutant protein has about a 1000-fold reduction in PPIase-like activity [14]. After 24 h MTS assay was used to determine cells proliferation.

Cell culture and treatments. H446 cells were cultured in 1640 medium with 10% fetal bovine serum (FBS) and 4.5 g/L glucose, 10 mM Hepes, and 1.0 mM sodium pyruvate at 37 °C with 5% CO_2. 3×10^5 H446 cells were plated equally on 35-mm tissue culture dishes. After an overnight incubation to allow the cells to adhere, cells were starved with medium containing no FBS for 24 h. Then cells were treated with CypA (10 nM) for 5, 10, 15, 30, or 60 min or different concentration of CypA at 0, 0.01, 0.1, 0.5, 1, 10 nM for 10 min. After washed with ice-cold PBS once, cells were lysed with ice-cold lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, and 1 mM PMSF) for 30 min on ice. Cell lysates were collected after centrifugation at 12,000 rpm for 10 min at 4 °C.

Western blot analysis. Total cellular proteins were separated with 12% SDS–PAGE and then transblotted for 2 h onto PVDF membrane (Amersham Biosciences, Arlington Heights, IL). After block with 3% BSA, the membrane was probed with ERK1/2 , JNK, p38, p-ERK 1/2 , p-JNK, and p-p38 at 4 °C overnight and then washed three times with 0.1% Tween 20-TBS and incubated in a horseradish peroxidaselinked secondary anti-rabbit or anti-mouse (1:5000) for 1 h at room temperature. The membrane was washed three times with 0.1% Tween 20-TBS and the immunoactive bands were detected by using an enhanced chemiluminescent (ECL) plus reagent kit.

Results

CypA and its receptor CD147 are both overexpressed in lung cancer cells

To investigate the expression levels of CypA and its receptor CD147 in human lung cancer, we quantified and compared the mRNA levels of CypA and CD147 in four human lung cancer cell lines (H1299, H446, A549 and SPC-A1) as well as in Hs888lu cells as nontumor cell controls. The mRNA level was quantified by real-time RT-PCR using specifically designed primers for CypA and CD147. GAPDH was used as the housekeeping gene to avoid variation between different samples. The threshold

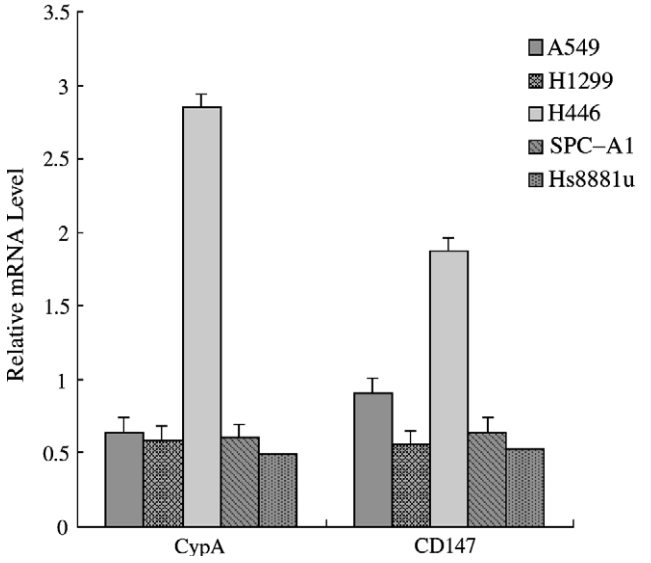

Fig. 1. Expression of CypA and CD147 mRNA in four kinds of lung cancer cells and Hs888lu cell. The mRNA level of each sample for each gene was normalized to that of GAPDH mRNA. Relative mRNA level was presented as $2^{[Ct(\_GAPDH)-Ct\ (gene\ of\ interest)]}$. All data shown are means ± SEM of three separate experiments ($P < 0.05$).

Table 1
Primer sequences of each gene for real-time PCR

Gene	GenBank No.	Forward primer	Reverse primer
CypA	NM_021130	GTCAACCCCACCGTGTTCTTC	TTTCTGCTGTCTTTGGGACCTTG
CD147	AB085790	CCATGCTGGTCTGCAAGTCAG	CCGTTCATGAGGGCCTTGTC
GAPDH	NM_002046	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC