Original Article

# Expression of large tumour suppressor (LATS) kinases modulates chemotherapy response in advanced non-small cell lung cancer

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**Background:** The Hippo signalling pathway plays an important role in regulating organ size and cell proliferation. Down-regulation of large tumour suppressor (LATS) protein homologs *LATS1* or *LATS2* has been found in lung cancer. *LATS1* and *LATS2* are the core components of the Hippo signalling pathway. *LATS1* and *LATS2* share some conserved structural features and exhibit redundant biological functions. The aim of this study was to dissect the interaction between these two homologs.

**Methods:** In lung adenocarcinoma (AD) cells, protein expression of *LATS1* and *LATS2* were determined by western blotting; cell viability and apoptosis were measured by MTT and annexin V staining after treatment with cisplatin; subcellular distributions of *LATS* proteins were determined by immunofluorescence microscopy; *LATS2* expression was modulated by shRNA-mediated knockdown or ectopic expression in cancer cell lines.

**Results:** Manipulation of the expression of these two *LATS* kinases influenced cisplatin response in advanced lung AD cell lines. High *LATS2*-to-*LATS1* ratio in H2023 cells was associated with cisplatin resistance, while low *LATS2*-to-*LATS1* ratio in CL1-0 and CL83 cells was associated with sensitivity to cisplatin. Manipulating the *LATS2*-to-*LATS1* ratio by *LATS2* over-expression in CL1-0 and CL83 rendered them resistant to cisplatin treatment, whereas *LATS2* knockdown in H2023 alleviated the *LATS2*-to-*LATS1* ratio and sensitized cancer cells to cisplatin exposure.

**Conclusions:** Our data suggested that the ratio of expression of *LATS* kinases played a role in the modulation of cisplatin sensitivity in advanced lung AD, and targeting of *LATS* proteins as a novel therapeutic strategy for lung AD deserves further investigation.

**Keywords:** Hippo; large tumour suppressor (LATS); lung cancer

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## Introduction

Lung cancer ranks the most common cancer and is also the leading cause of cancer mortality in most parts of the world (1). Non-small cell lung cancer (NSCLC) accounts for more than 80% of pulmonary tumours, in which adenocarcinoma (AD) is the predominant subtype (2). Lung cancer has been reported to be heterogeneous in terms of histological, biological characteristics, and response to chemotherapy, targeted therapy, and checkpoint inhibitor blockade (CIB) immunotherapy (3). Heterogeneity of responses to targeted therapy is largely explained by the discovery of "druggable" driver mutations for targeted and personalized treatments, such as epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) (e.g., erlotinib, gefitinib, and afatinib) targeting sensitizing mutations in the *EGFR* gene, and anaplastic lymphoma kinase (*ALK*) inhibitors targeting *EML4-ALK* gene rearrangements (e.g., crizotinib and ceritinib and alectinib) (4). Lung cancers that showed good response to CIB immunotherapy have several molecular biomarker predictors including expression of the target PD-L1, total tumor mutation burden (TMB) leading to high neo-antigen expression and a high degree of mutation clonality (5).

Despite the significant breakthrough in molecular targeted treatment and immunotherapy, platinum-based chemotherapy is still one of the first-line treatments for advanced stage lung cancer and also remains the mainstay of care for patients developing resistance to targeted agents (6,7). The most commonly used platinum-based regimens are cisplatin plus gemcitabine, pemetrexed, taxanes or vinorelbine ("platinum-doublet" chemotherapy) (8). The combination of cisplatin and pemetrexed is considered as a standard of care treatment option for patients with non-squamous NSCLC (AD and large-cell carcinoma) (9). However, only less than half of lung cancer patients demonstrated good response to platinum-doublet chemotherapy. Thus, a major issue in the treatment of advanced stage NSCLC is to identify biomarkers that could predict therapeutic response to platinum-doublet chemotherapy.

The human large tumour suppressor (LATS) proteins, consisting of *LATS1* and *LATS2*, were identified as core components of the Hippo signalling pathway (10). The major function of the *Hippo* pathway is the regulation of organ size by coordinating cell proliferation, cell death and cell differentiation (11). De-regulation of this pathway has been shown to induce tissue over-growth (11) and this occurs in some types of human carcinomas, including lung, colorectal, breast and liver cancers (12). The upstream regulation of *LATS1/2* kinases is complex and is not fully understood. In the canonical *Hippo* pathway, activated *MST1/2* (mammalian sterile 20-like kinases 1 and 2) is associated with *SAV1* phosphorylation of *LATS1/2* and *MOB1*, leading to the formation of *LATS-MOB1* complex. Within this complex, *LATS1/2* kinases are fully activated by phosphorylation on both T-loop and hydrophobic sites. The resulting activated *LATS* kinases interact with and phosphorylate *YAP* (yes-associated protein)/*TAZ* (transcriptional co-activator with PDZ-binding motif), rendering cytoplasmic sequestering and subsequent degradation of these oncogenic transcriptional co-factors (11,13).

As homologs, *LATS1* and *LATS2* share some conserved features including the common composition of a C-terminal kinase domain, one protein-binding domain, two *LATS* conserved domains, an ubiquitin-associated domain and at least one PPxY motif which can interact with proteins possessing WW domain (10). However, each type of *LATS* kinase exhibits unique domains which may contribute to their distinct functions: *LATS1* has a proline-rich P-stretch (14), while *LATS2* shows repeats of alternating proline-alanine residues (PAPA repeat) (15). The down-regulation of *LATS1* or *LATS2* has been found in breast cancer (16), prostate cancer (17), colorectal cancer (18), gastric cancer (19), hepatic carcinoma (20) and certain subtypes of ovarian cancer (21). In NSCLC, decreased expression of *LATS1* (22) or *LATS2* (23) has been reported to correlate with poor prognosis in terms of shorter overall survival.

Not much research has investigated into the effects of *LATS* kinases on chemo-sensitivity in NSCLC. Furthermore, since *LATS1* and *LATS2* share high similarity in protein structure and exhibit redundant roles in the Hippo pathway, studies dissecting the interaction or regulation between these two homologs are needed. The hypothesis of this study was that changes in the relative expression of *LATS1* and *LATS2* could affect the chemotherapeutic response of lung cancer cells. Thus, we set out to explore if manipulation of the relative expression of *LATS* kinases would modulate cisplatin chemotherapy response in advanced stage lung AD.

## Methods

### *Human lung AD cell lines*

Ten AD cell lines were cultured in RPMI 1640 (Gibco, USA) supplemented with 1% Penicillin-Streptomycin (Gibco, USA) and 2.5% or 10% fetal bovine serum

(Gibco, US). The ten lung AD cell lines used in this study were HKULC-2 (24), FA31 and FA98 established from pleural fluids of lung AD subjects, developed by the Lam lab; H2023, H1975 and H1650 from JD Minna M.D., University of Texas Southwestern Medical Center at Dallas, USA. CL1-0, CL83, H3255 and PC9 were gifts from PC Yang, M.D., National Taiwan University.

CL1-0, CL83, H2023, FA98 and HKULC-2 were *EGFR* wild-type cell lines; while the remaining five cell lines harboured mutations in *EGFR* gene (H3255 and FA31 with L858R point mutation, H1650 and PC9 carried deletions at exon 19 and H1975 has both *EGFR* L858R and T790M mutations). All cell lines were maintained in a humidified incubator at 37% with 5% $CO_2$.

### *Plasmids and transfection*

The pCMV6 full-length human *LATS2* expression vector tagged with MYC/DDK was obtained from OriGene, USA. The *LATS2* sequences were confirmed by DNA sequencing. Transfection of cells with this *LATS2* plasmid vector was performed via Lipofectamine LTX&PLUS system (Invitrogen, USA). Briefly, cells were plated on 24-well plates on the day before transfection. For each well, 500 ng plasmid DNA in complex with Lipofectamine reagents was added for 24 hours. Culture media containing DNA-Lipofectamine complexes were replaced with G418-contained media in order to select cells that have *LATS2* plasmid stably integrated into cell genome. The expression efficiency of this *LATS2* plasmid was determined by protein expression assays. Cells transfected with empty vector was used as control.

### *Short hairpin RNA (shRNA) and infection*

The lentiviral transduction particles which contained control shRNA or *LATS2*-targeted shRNAs cloned into the pLKO.1-puro vector (Sigma-Aldrich, USA) were utilized to establish stable knockdown of *LATS2* in lung AD cells. The infection protocol was provided by the manufacturer. For each cell line, the optimal multiplicity of infection was tested empirically, and the transduced cells were selected through puromycin treatment. The surviving cell colonies were harvested to test for knockdown efficiency by means of western blotting.

### *Cisplatin treatment and cell viability assay*

Cisplatin was purchased from Sigma-Aldrich, and was dissolved in 0.9% NaCl and kept as a stock solution (10 mM) at −80 ℃. Cells were seeded in 96-well plates at appropriate density, and then exposed to different concentrations of cisplatin solution (0, 1, 10, 20, 50, 100 μM) for 72 hours. 3-(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution was added to each well for testing cell viability based on manufacturer instructions (Sigma-Aldrich, USA).

### *Immunofluorescence staining*

In brief, cells were plated on coverslips and, after proper treatment, cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). After permeabilization with 0.1% Triton X-100 in PBS and blocking, cells were incubated with rabbit anti-human *LATS2* (Cell Signaling, USA) and mouse anti-human *LATS1* (Santa Cruz, USA) antibodies, followed by incubation with AlexaFluor 488- and 594-conjugated anti-rabbit and anti-mouse IgG (Molecular Probes, USA), respectively. DNA was counter-stained with DAPI (Invitrogen, USA). Images were obtained using fluorescence microscopy (Nikon Eclipse Ni-U, Japan).

### *Western blotting and antibodies*

Immunoblotting experiments were conducted as described before (23). Primary antibodies to *LATS1*, *LATS2*, cleaved caspase-3, caspase-3 and *p53* were obtained from Cell Signaling, USA. β-actin (Sigma-Aldrich, USA) was used as a loading control. The band intensity of each target was quantified with Image J software.

### *Statistical analysis*

Graphics were analyzed with Image J software and statistical analysis was carried out using GraphPad Prism software. Data were presented as mean ± SEM, unless stated otherwise. Differences between groups were estimated using two-tailed unpaired Student's *t* test. A probability level of 0.05 was used to determine statistical significance.

## Results

### *The expression of LATS2 and LATS1 at baseline in lung AD cell lines*

The basal expression levels of *LATS1* and *LATS2* were

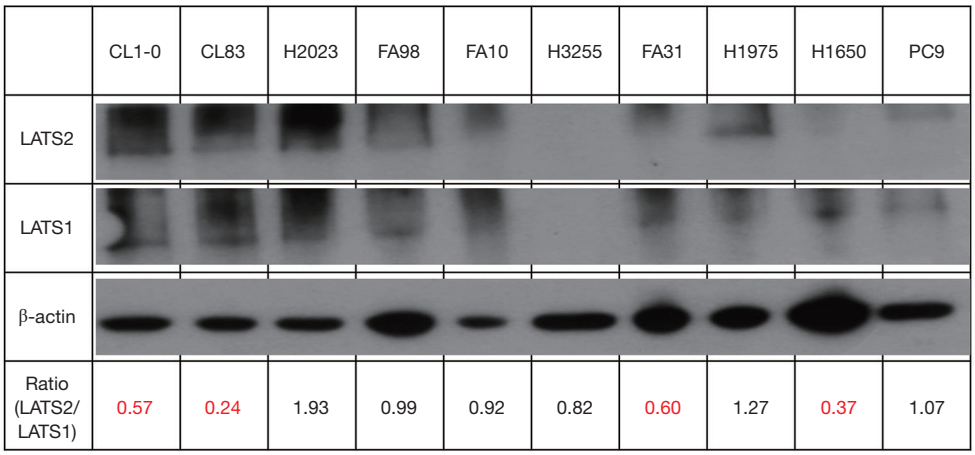



**Figure 1** Baseline protein expressions of *LATS1* and *LATS2* in different lung AD cell lines. The *LATS1* and *LATS2* protein expressions were determined by Western blotting (n=3, assays were done in triplicates for each cell line).

assayed in the ten lung AD cell lines. As shown in *Figure 1*, these cell lines expressed *LATS1* protein at a relatively consistent level except for H3255, whereas the expression levels of total *LATS2* kinase were quite diverse in different cell lines. In some cell lines, such as CL1-0, CL83, FA31 and H1650, *LATS2* expression levels were much lower than the levels of *LATS1*. We defined this group of cell lines as the "low" *LATS2*-to-*LATS1* group. On the other hand, H2023 and H1975 cell lines were categorised as the "high" *LATS2*-to-*LATS1* group, since these cells displayed more *LATS2* kinase than *LATS1*. For remaining cell lines (FA98, HKULC-2, H3255 and PC9), these two *LATS* kinases were expressed at comparable levels.

### *The effect of alterations in LATS2-to-LATS1 expression ratios on cell viability after cisplatin treatment*

Low *LATS2*-to-*LATS1* cell lines CL1-0 and CL83, were transfected with *LATS2*-over-expression plasmid vectors for ectopic expression of *LATS2* in these cells (*Figure 2A,B*). After selecting and expanding successfully transfected cell clones, different concentrations of cisplatin solution (0–100 μM) were added to both the parental and LATS2-transfected cells. LATS2 over-expression rendered both cell lines more resistant to cisplatin treatment, as indicated by more viable cells after cisplatin addition and the higher $IC_{50}$ values in LATS2-transfected cells with larger LATS2-to-LATS1 ratios (*Figure 2A,B*).

At the same time, we silenced *LATS2* expression by infecting high *LATS2*-to-*LATS1* H2023 cells with viral particles-containing control or *LATS2*-specific shRNA (*Figure 2C*). *LATS2* knockdown resulted in a decrease in the *LATS2*-to-*LATS1* ratio and sensitized H2023 cells to cisplatin. The $IC_{50}$ concentration of cisplatin dropped dramatically in *LATS2*-silenced H2023 cells compared with H2023 cells with control shRNA (*Figure 2C*).

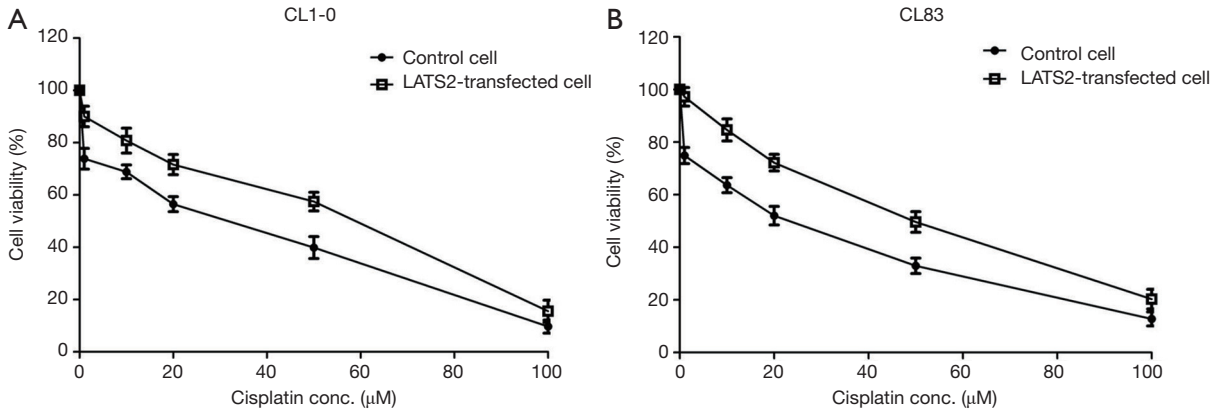
### *Changes in LATS2-to-LATS1 ratios modulated cisplatin-induced apoptosis in lung AD*

Apart from cell viability, apoptosis in response to cisplatin treatment was altered after manipulation of *LATS2*-to-*LATS1* ratios. For low *LATS2*-to-*LATS1* cell lines CL1-0 (*Figure 3A*) and CL83 (*Figure 3B*), the increase of *LATS2*-to-*LATS1* ratios by over-expressed *LATS2* quantitatively inhibited cisplatin-induced apoptosis, since the percentage of apoptotic cells as well as the cleavage of caspase-3 (*Figures 3C,D*) were both significantly reduced in *LATS2*-overexpressed CL1-0 and CL83 cells after cisplatin addition. On the contrary, silencing of *LATS2* in high *LATS2*-to-*LATS1* H2023 cells promoted apoptosis triggered by cisplatin (*Figure 3E*), accompanied by the enhancement of caspase-3 activation (*Figure 3F*).

### *The influence of LATS2 on LATS1 subcellular location after cisplatin treatment*

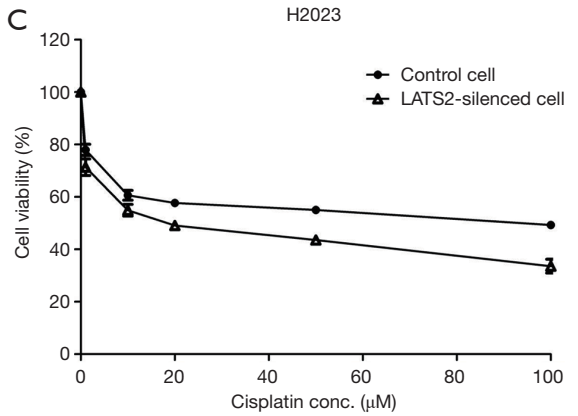
We studied subcellular location of *LATS* kinases and found that *LATS2* kinase was ubiquitously present in both cytoplasm and nucleus, whereas the majority of *LATS1*




| CL83 | Control cell | LATS2-transfected cell |
|---|---|---|
| $IC_{50}$ (μM) | 15.80±1.613 | 42.53±3.923 |

| CL83 | Control cell | LATS2-transfected cell |
|---|---|---|
| $IC_{50}$ (μM) | 13.08±1.972 | 45.60±9.215 |

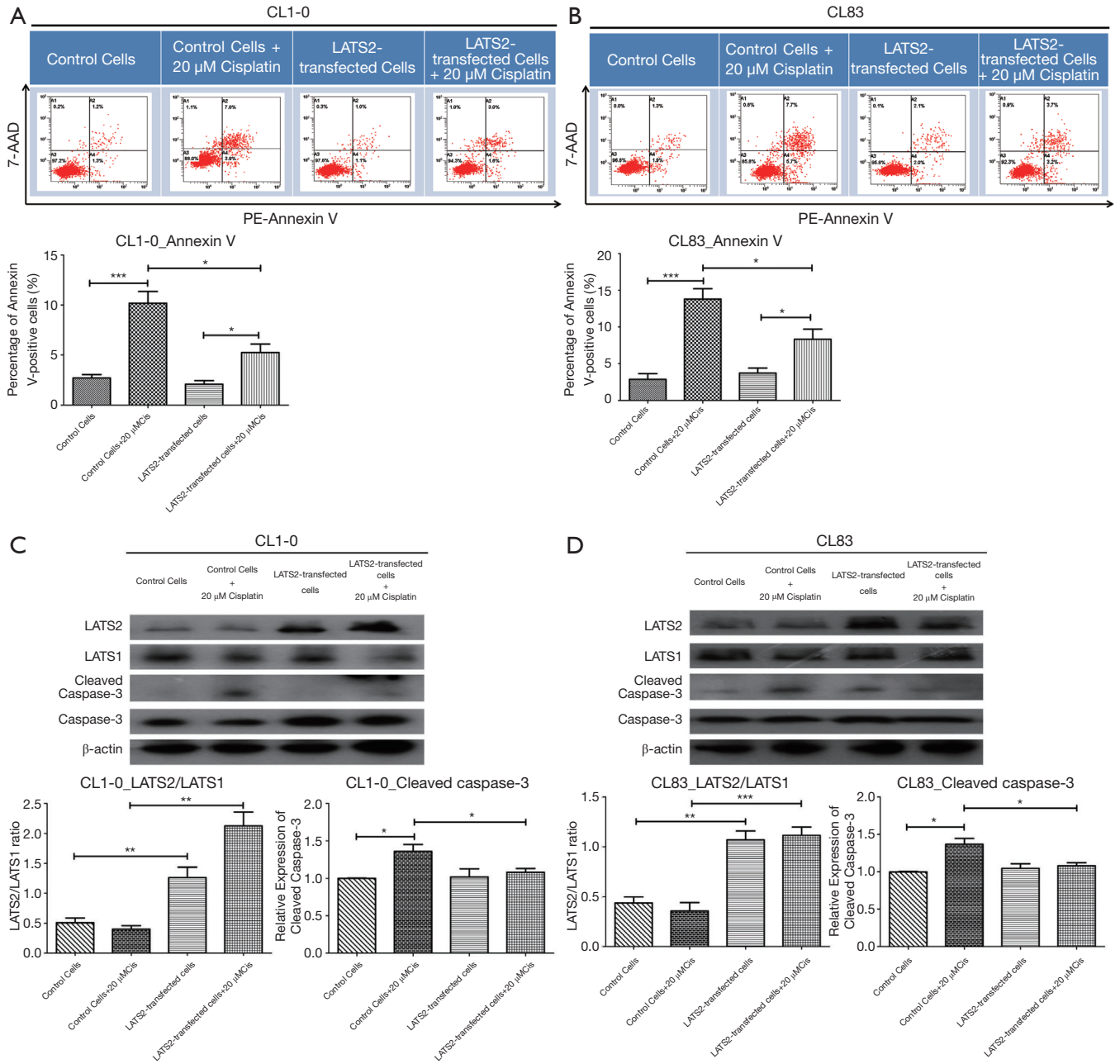



| CL83 | Control cell | LATS2-transfected cell |
|---|---|---|
| $IC_{50}$ (μM) | 92.56±16.69 | 17.11±2.054 |

**Figure 2** The effect of manipulation of *LATS2*-to-*LATS1* ratios on cisplatin sensitivity in lung AD cell lines. (A) CL1-0 cells were transfected with vectors containing *LATS2* cDNA (LATS2-transfected cells) or empty vector (control cells). Cell viability was measured by MTT assay (n=4); (B) the same procedures as in (A) were performed with CL83 cells (n=4); (C) H2023 cells were transfected with viral particles-containing control shRNA or *LATS2* shRNA for 24 hours. After selection, total protein was extracted, and the expression levels of *LATS* kinases in control cells and *LATS2*-shRNA cells were measured (n=3). Subsequently, both control and LATS2-silenced cells were treated with 0–100 μM cisplatin solution for 72 hours, and cell viability was tested with MTT assay (n=4). AD, adenocarcinoma; LATS, large tumour suppressor.

kinase was present in the nucleus with sparse staining in the cytoplasm (*Figure 4*). In two low *LATS2*-to-*LATS1* cell lines CL1-0 and CL83, addition of cisplatin enhanced 2 to 3-folds of *LATS1* translocation from cytoplasm to nucleus (*Figures 4A*,*B* upper panels; *Figure 4D* left and middle panels). However, this phenomenon was absent in high *LATS2*-to-*LATS1* cell lines H2023 (*Figure 4C* upper panel, *Figure 4D* right panel). It was notable that H2023 was more resistant to cisplatin than CL1-0 and CL83 (*Figure 2A*,*B*). Furthermore, *LATS2* over-expression in low *LATS2*-to-*LATS1* cells (CL1-0 and CL83) mitigated cisplatin-induced nuclear translocation of *LATS1* kinase (*Figure 4A*,*B* lower panels; *Figure 4D* left and middle panels). For *LATS2*-silenced H2023 cells which were more sensitive to cisplatin compared with control H2023 cells (*Figure 2C*), more *LATS1* was able to translocate into nucleus (*Figure 4C* lower panel; *Figure 4D* right panel). These findings implied that *LATS2* kinase could regulate the cellular localization of its



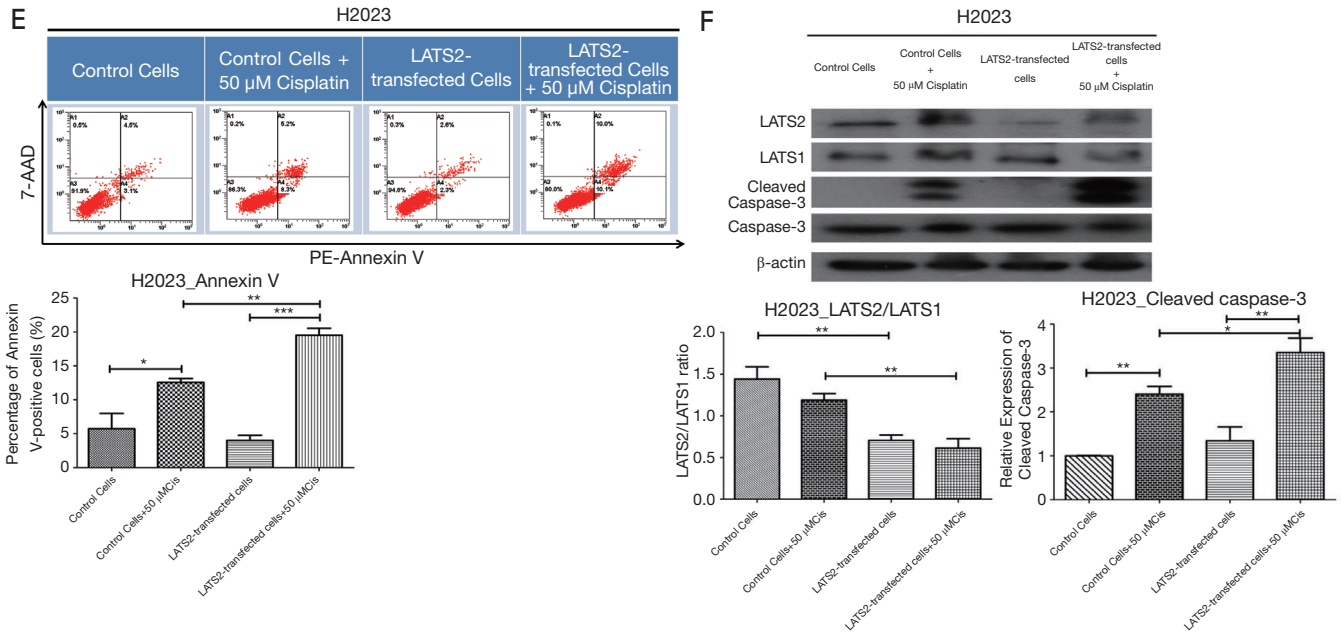


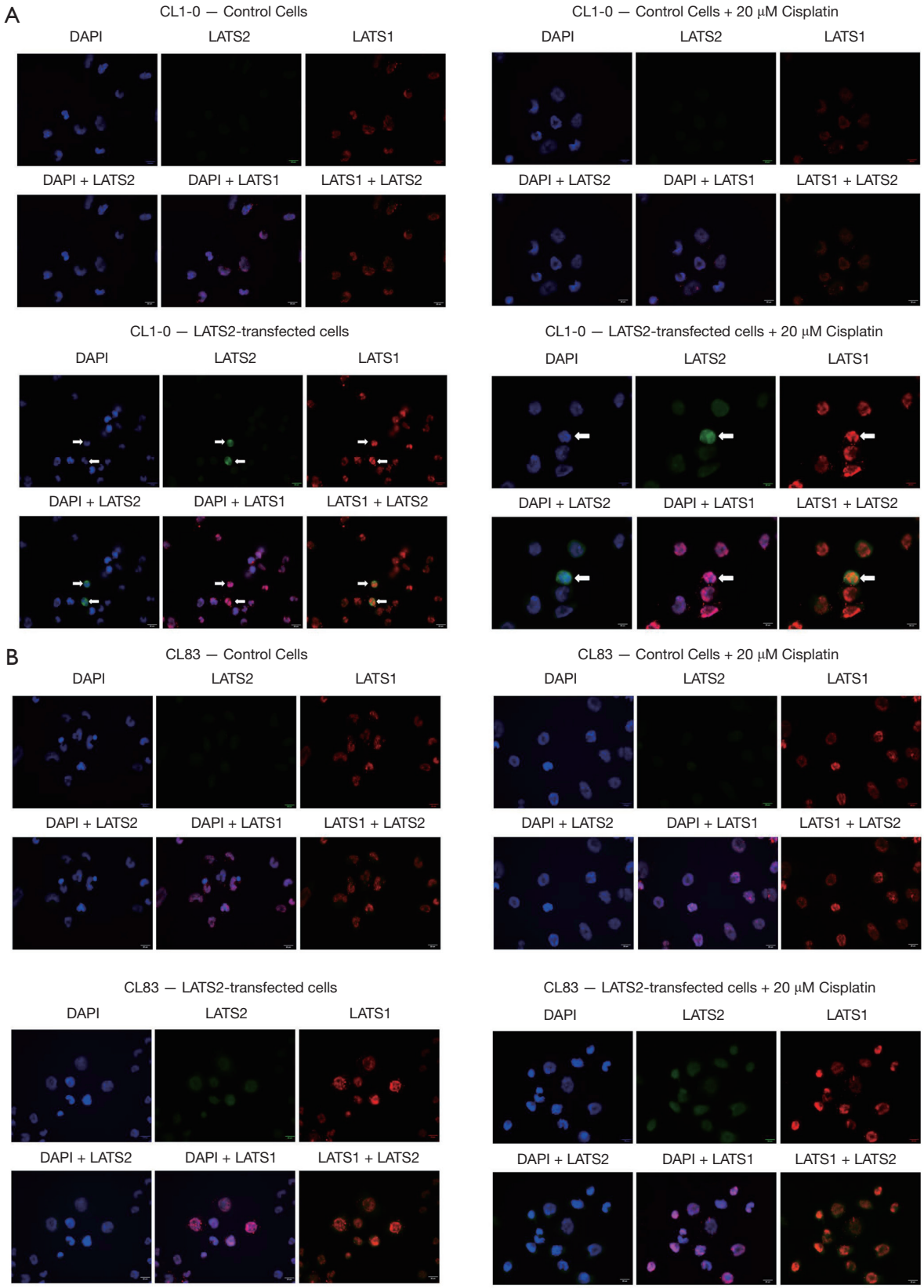

**Figure 3** Changes in *LATS2*-to-*LATS1* ratios modulated cisplatin-induced apoptosis in lung AD cell lines. (A) Both control and *LATS2*-transfected CL1-0 cells were treated with or without 20 μM cisplatin for 72 hours. The levels of cisplatin-induced apoptotic cells were measured by Annexin V assay (n=4); (B) CL83 cells were treated as described in (A); (C) the expression levels of indicated proteins in control and *LATS2*-transfected CL1-0 and (D) CL83, were analysed by Western blotting (n=4); (E) apoptotic levels were determined in control and *LATS2*-silenced H2023 cells with 50 μM cisplatin treatment for 72 hours (n=4); (F) the expression levels of indicated proteins in control and *LATS2*-silenced H2023 were analysed by Western blotting (n=4). *, P<0.05; **, P<0.01; ***, P<0.001. AD, adenocarcinoma.

homolog *LATS1* and nuclear translocation of *LATS1* kinase might be related to cisplatin sensitivity.

## Discussion

This study was the first demonstration that manipulation of expression of *LATS* kinases was able to regulate sensitivity to cisplatin in lung AD cell lines. This is important because cisplatin-based doublet chemotherapy is a commonly used first-line treatment for advanced NSCLC patients. The well-known mechanism underlying the anti-neoplastic effects of cisplatin is to generate excess and irreparable DNA lesions, thus committing cells to cellular senescence or mitochondrial pathway of apoptosis (25). There is recent evidence that, in addition to its genotoxic activity, cytoplasmic cisplatin also exerts certain cytotoxic functions through accumulation of reactive oxygen species and establishment of endoplasmic reticulum stress (26,27).

Although platinum-based chemotherapy could be effective in the initial control of advanced stage lung cancer disease, platinum drug administration is dosage limited and could not be used for maintenance chemotherapy due to potential cumulative toxicity. Some NSCLC also become resistant to chemotherapy. If there is any gene expression characteristics that predict platinum chemotherapeutic response or resistance or if that gene expression could be modulated, this may help in modifying the platinum dosage schedule and hence could reduce its potential cumulative adverse effects. Cisplatin resistance is usually multi-factorial, with potential involvement from either or both tumour cell-intrinsic and tumour cell-extrinsic (stem cell, stromal cell, or immune cell-related) pathways (25,28). In this study, we have shown that the high *LATS2*-to-*LATS1* ratio was associated with cisplatin resistance in lung AD cells. High *LATS2*-to-*LATS1* H2023 cells were more resistant to cisplatin than low *LATS2*-to-*LATS1* cells (CL1-0 and CL83) (*Figure 2*). Furthermore, the increase in the *LATS2*-to-*LATS1* ratio by *LATS2* over-expression in CL1-0 and CL83 rendered cancer cells more insensitive to cisplatin treatment, whereas *LATS2* knockdown in H2023 alleviated the *LATS2*-to-



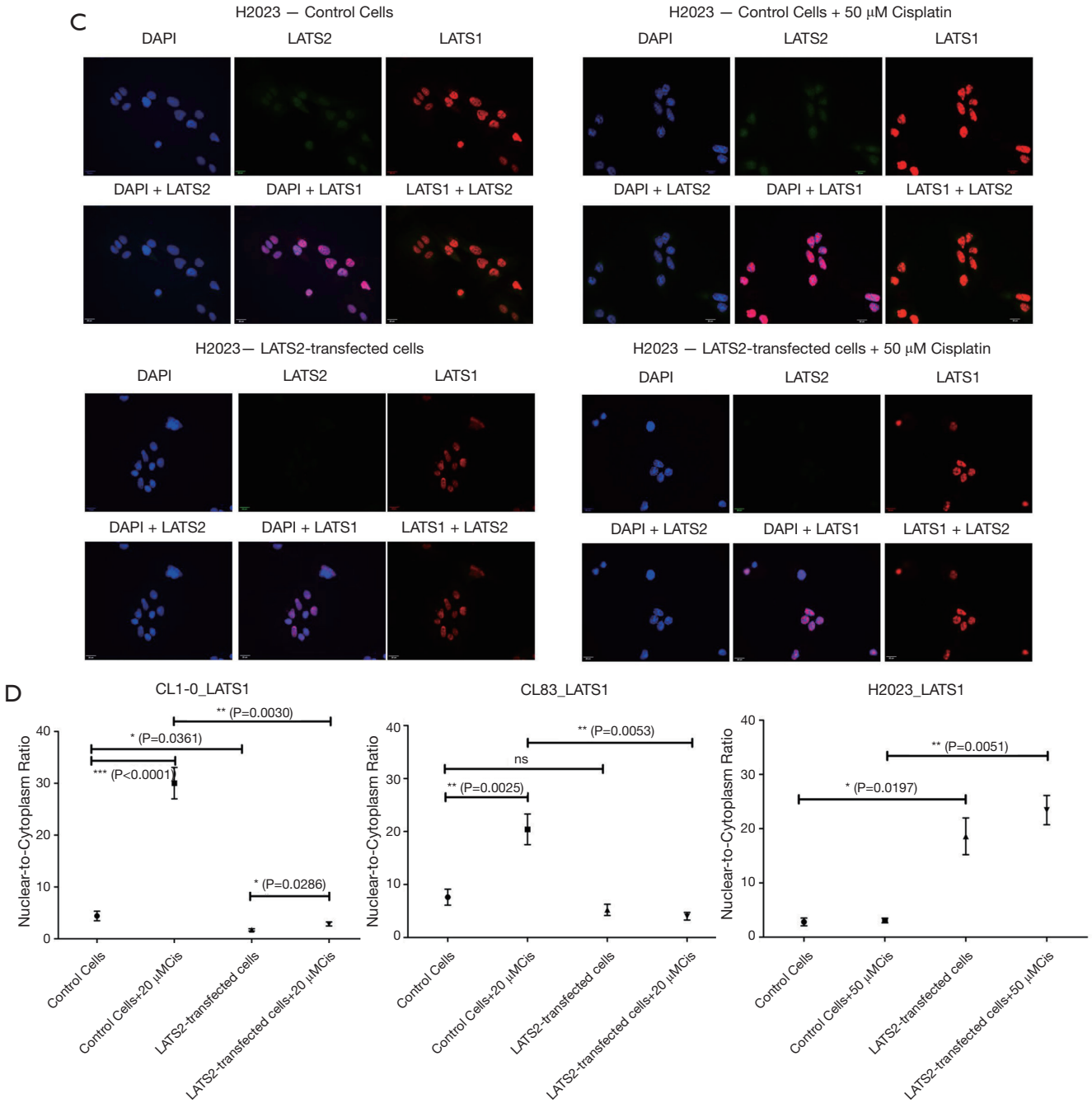



**Figure 4** *LATS2* kinase regulated the subcellular localisation of *LATS1* kinase, and nuclear translocation of *LATS1* in lung AD cell lines. (A,B) Both control and *LATS2*-transfected CL1-0 and CL83 cells were treated with or without 20 μM cisplatin for 72 hours. After treatment, cells were immunofluorescence stained with *LATS1*-specific or *LATS2*-specific antibodies conjugated with distinct fluorescent probes (n=3); (C) the same method with 50 μM cisplatin was used in both control and LATS2-silenced H2023 cells (n=3). (A,B,C) Magnification, 40×. Scale bar 20 μm; (D) quantitative analysis of nuclear and cytoplasmic ratio of *LATS1* in three lung AD cell lines. *, P<0.05; **, P<0.01; ***, P<0.001. AD, adenocarcinoma.

*LATS1* ratio and actually sensitized cancer cells to cisplatin administration. Besides, according to latest publicly available TCGA data on the *LATS1* and *LATS2* expressions in lung cancer, *LATS2* alone is significantly down-regulated in lung tumour tissue when compared with adjacent normal tissue, in both lung AD and SCC (*Figure S1*).

The results from this study also supported a potential role of *LATS2* kinase in the regulation of *LATS1* localization. In low *LATS2*-to-*LATS1* cells, the addition of cisplatin promoted nuclear translocation of *LATS1*. After cisplatin treatment, however, the cellular distribution of *LATS1* kinase did not change in high *LATS2*-to-*LATS1* cells. In addition, over-expression of *LATS2* in low *LATS2*-to-*LATS1* cells considerably inhibited the nuclear entry of *LATS1* kinase and moderated pro-apoptotic effects of cisplatin. The situation was reversed when *LATS2* was silenced in high *LATS2*-to-*LATS1* cells. The reduction in the *LATS2*-to-*LATS1* ratio not only enabled more *LATS1* to enter into the nucleus but also encouraged anti-neoplastic effects to take place in cancer cells. Little is known regarding the mechanisms that mediate the cellular distribution of *LATS* kinases. Previous studies found that *LATS* kinases together with other hippo pathway members were localized to centrosomes to modulate the various steps of cell cycle including centrosome numbering, spindle formation, chromosome segregation and cytokinesis (29-33), although the differential aptitudes of *LATS* kinases to become localized and activated in centrosomes are not fully understood. Differential functions of *LATS1* and *LATS2* expression have been proposed in studies by Furth *et al.* which suggested that the reduced expression of either *LATS1* or *LATS2* may re-wire breast cancer signalling networks (34). Lower *LATS1* but not *LATS2* mRNA expression has been found to correlate with poor survival in NSCLC (35).

The strength of our study is that manipulation of *LATS2/LATS1* was shown to modulate sensitivity towards platinum chemotherapy and was associated with survival in NSCLC, with mechanistic implication of the translocation of LATS kinases in different subcellular locations. There were, however, limitations. First, this study was based on lung cancer cell lines. Direct manipulation of *LATS2/LATS1* may not be possible in lung cancer patients but at least the assays of *LATS2/LATS1* ratio in lung tissues or metastatic specimens such as pleural fluid, could be informative as to the potential sensitivity of the disease towards platinum-based chemotherapy. Moreover, this study focused on *LATS2/LATS1* expression and yet there could be collateral or downstream signalling pathways involved in chemotherapeutic sensitivity. Further research is required to clarify the detailed mechanisms concerning the regulation of subcellular localization of LATS kinases as well as their interactions.

## Conclusions

In summary, our data provided evidence that the relative expression of *LATS* kinases played a role in the modulation of cisplatin sensitivity in advanced lung AD. This modulation might be ascribed to the differential subcellular distribution of *LATS1* which was influenced by *LATS2* expression. The findings of this study warrant further translational investigation on the use of *LATS2*-to-*LATS1* ratio as a potential biomarker or a therapeutic target.

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## Footnote

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tlcr.2020.03.26). JDM received royalties for licensing human tumor and related lines from the National Institutes of Health (NIH) and University of Texas Southwestern Medical Center. JDM's lab at UTSW receives research support from licensing fees for the distribution of the human lines. The other authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was approved by the Institutional Review Board/Ethics Committee of the Hong Kong University/Hong Kong Hospital Authority Hong Kong West Cluster (HKU/HA HKWC IRB/EC UW 16-104).



 

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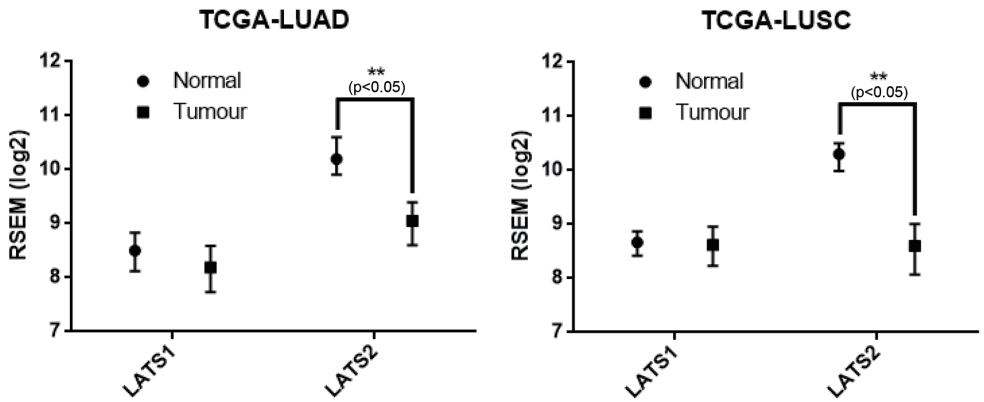


**Figure S1** Expression levels of *LATS1* and *LATS2* in resected lung tumor. *LATS1* and *LATS2* expressions based on RNA-seq data from The Cancer Genome Atlas (TCGA) datasets in lung adenocarcinoma and squamous cell carcinoma. Comparisons were made between tumour and adjacent normal tissues; data was sorted by GDAC FireBrowse.