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Aberrant large tumor suppressor 2 (*LATS2*) gene expression correlates with *EGFR* mutation and survival in lung adenocarcinomas

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Abstract

Background—Large tumor suppressor 2 (*LATS2*) gene is a putative tumor suppressor gene with potential roles in regulation of cell proliferation and apoptosis in lung cancer. The aim of this study is to explore the association of aberrant *LATS2* expression with *EGFR* mutation and survival in lung adenocarcinoma (AD), and the effects of *LATS2* silencing in both lung AD cell lines.

Methods—*LATS2* mRNA and protein expression in resected lung AD were correlated with demographic characteristics, *EGFR* mutation and survival. *LATS2*-specific siRNA was transfected into four *EGFR* wild-type (WT) and three *EGFR* mutant AD cell lines and the changes in *LATS2* expression and relevant signaling molecules before and after *LATS2* knockdown were assayed.

Results—Fifty resected lung AD were included (M:F = 23:27, smokers:non-smokers = 19:31, *EGFR* mutant:wild-type = 21:29) with *LATS2* mRNA levels showed no significant difference between gender, age, smoking and pathological stages while *LATS2* immunohistochemical staining on an independent set of 79 lung AD showed similar trend. *LATS2* mRNA level was found to be a significant independent predictor for survival status (disease-free survival RR = 0.217; $p = 0.003$; Overall survival RR = 0.238; $p = 0.036$). siRNA-mediated suppression of *LATS2* expression resulted in augmentation of *ERK* phosphorylation in *EGFR* wild-type AD cell lines

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Conflict of interest statement

All the authors in this manuscript declare no conflict of interest that could inappropriately influence or bias this piece of work.

with high basal *LATS2* expression, discriminatory modulation of *Akt* signaling between *EGFR* wild-type and mutant cells, and induction of *p53* accumulation in AD cell lines with low baseline *p53* levels.

Conclusions—*LATS2* expression level is predictive of survival in patients with resected lung AD. *LATS2* may modulate and contribute to tumor growth via different signaling pathways in *EGFR* mutant and wild-type tumors.

Keywords

Lung cancer; Large tumor suppressor 2 gene; Gene expression; Gene silencing; EGFR signaling

1. Introduction

LATS2 (large tumor suppressor 2), one of the two human homologues of *Drosophila* warts, is a putative tumor suppressor gene which encodes for a serine/threonine kinase [1]. As a component in the Hippo signaling pathway, *LATS2* kinase plays a critical role in controlling organ size development and in coordinating cell proliferation and apoptosis [2,3]. As a putative tumor suppressor gene, *LATS2* displays multiple mechanisms of actions in different cancer cell types, including cell cycle regulation by controlling G1/S and G2/M transition [4,5], induction of apoptosis by down-regulating anti-apoptotic proteins Bcl-2 and Bcl-X_L [6], as well as maintenance of mitotic fidelity and genetic stability by interacting with other regulators of cell division, such as *p53* [7] and Aurora kinases [8–10].

Dysregulation of *LATS2* functions has been found in different types of tumors. The chromosomal location of *LATS2* is mapped to 13q11–q12 where there is frequent loss of heterozygosity [11]. In breast cancer, *LATS2* mRNA expression was down-regulated by promoter hypermethylation and this alteration was associated with large tumor size, high rate of metastasis and estrogen receptor and progesterone receptor negativity [12]. *LATS2* may also play a role in the development of prostate cancer based on findings that reduced *LATS2* expression occurred in prostate tumors and *LATS2* negatively modulated androgen receptor-regulated gene transcription [13]. In malignant mesothelioma (MM), *LATS2* was found to be inactivated in MM cells and this inactivation will lead to deregulated cell growth by allowing constitutive activation of the downstream transcription factor of *LATS2* in the Hippo pathway, YAP [14,15]. The expression of *LATS2* exhibits suppressive effects on mesothelioma cells.

In non-small cell lung cancer (NSCLC), occasional information has been reported on the effects of aberrant expression of *LATS2* except its being one potential targets for microRNA-135b action to promote lung cancer metastasis [16]. *LATS2* mutations are rare [17,18] but other mechanisms have been reported to cause down-regulation of its expression such as promoter hypermethylation [19] and micro-RNA regulation [20,21]. The relation between reduced *LATS2* expression and lung cancer progression, and the underlying mechanisms remain unknown. In addition, we have previously found from expression profiling experiments that *LATS2* showed differential expression between pulmonary adenocarcinomas with wild-type *EGFR* and ones bearing *EGFR* mutations at exons 18–21 [22]. In this study, we further validated the differential expression of *LATS2* in lung adenocarcinoma (AD) tissues in relation to *EGFR* mutation status, as well as other clinicopathological factors including as smoking history and survival status. Furthermore, we silenced *LATS2* expression by siRNA inference in several lung adenocarcinoma cell lines and examined *LATS2* knockdown effects on *EGFR* downstream signaling pathways, *Ras/Erk*, *PI3K/Akt*, as well as*p53* network.

2. Materials and methods

2.1. Human lung adenocarcinoma tissue

Newly diagnosed of lung adenocarcinoma patients were recruited prospectively before planned surgical resection. The protocol for lung cancer surgical specimen collection was approved by the Institutional Review Board Research Ethics Committee of the University of Hong Kong and Hong Kong Hospital Authority Hong Kong West Cluster. Resected lung adenocarcinoma (AD) tissues from Chinese patients were included in this study. Upon resection, these tissue samples were immediately submerged in RNA*later* RNA Stabilization Reagent (Qiagen), frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. An independent set of 79 archival paraffin blocks of lung adenocarcinomas with known patient demographics including age, gender and *EGFR* mutation status (but not smoking habits) collected successively at the Histopathology Laboratory of the Hong Kong and Sanatorium Hospital was used for immunohistochemical studies. All the tumor tissues used were collected from patients who underwent resection of lung tumors without prior treatment with EGFR tyrosine kinase inhibitors or any other form of anti-cancer treatment.

2.2. Human adenocarcinoma cell lines

Seven 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, USA). AD cell lines used in this study were HKULC-2, HKULC-4 [23], and H1648, H1650, H1975 and H2023 were from John D Minna M.D., University of Texas Southwestern Medical Center at Dallas; while PC9 was from PC Yang M.D., National Taiwan University. Three AD cell lines with *EGFR* mutations, which were H1975 with double mutations L858R and T790M, PC9 andH1650 bearing *EGFR* deletions at exon 19.

2.3. Direct sequencing for EGFR mutations

We utilized the historical standard for EGFR mutation testing, directing sequencing [24,25]. In order to enrich tumor cell content, we performed micro-dissection on formalin-fixed paraffin-embedded sections before DNA extraction. Then, exons 18–21 of EGFR were PCR-amplified by applying respective primers. Purified PCR product was analyzed by ABI 3730xl DNA analyzer and sequence data was reviewed by Sequence Scanner Software.

2.4. Real time PCR

Total RNA was extracted from frozen resected lung adenocarcinoma tissues by using the RNeasy kit (Qiagen, UK). After reverse transcription of total RNA via QuantiTect® kit (Qiagen, UK), 150 ng cDNA templates were used to detect *LATS2* mRNA expression by using quantitative real time PCR (RT-qPCR) with the SYBR Green I method (Qiagen, UK). The primer sequences for *LATS2* mRNA were: Forward, 5′-

TGGCACCTACTCCCACAG-3′, and Reverse, 5′-CCAAGGGCTTTCTTCATCT-3′ [26]. The ribosomal 18S gene was chosen as an internal control, and the primer sequences were: Forward, 5′-AGGAATTGACGGAAGGGCAC-3′, and Reverse, 5′-GGACATCTAAGGGCATCACA-3′. Thermal cycle conditions were: 95 °C for 5 min followed by 40 cycles of amplification at 95 °C for 15 s per cycle, 58 °C (*LATS2*) or 60 °C (18S) for 45 s and 72 °C for 45 s. The dissociation curve analysis was carried out to exclude non-specific primer dimers. The relative expression level was determined as $2^{-\Delta\Delta Ct}$ with relative to a reference sample (BS65.2N-KT, an immortalized normal bronchial epithelial cell line). The logarithmic values to base of two of these relative expression levels were used in the following statistical analysis.

2.5. Immunohistochemistry

Immunohistochemistry (IHC) of the *LATS2* protein was performed on formalin-fixed, paraffin-embedded sections of an independent set of 79 paraffin blocks of lung AD different from those used for real-time PCR assay above.

Antigen retrieval was conducted in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA, pH = 9) at 95 °C for 30 min. The goat polyclonal antibody against human *LATS2* (dilution 1:200; Santa Cruz, USA) or negative control mouse IgG1 (dilution: 1:200; Dako, Denmark) was incubated with the sections overnight at 4 °C. After treating with the rabbit anti-goat/mouse secondary antibodies (dilution 1:400; Dako, Denmark) respectively at 37 °C for 30 min, the specimens were stained with DAB substrate chromogen system (Dako, Denmark) for 5 min. Scoring of immunohistochemical staining was performed by independent pathologists (Ximing Tang and Ignacio I Wistuba) without knowing the clinical annotations of the specimens. *LATS2* expression H-scores based on the extension (0–100) and intensity (0, none; 1+, weak; 2+, moderate; and 3+, strong) of IHC staining was performed by two pathologists (X.T. and I.W.). If there is discrepancy between the two pathologists concerned, an independent third pathologist in the same laboratory who were not aware of the clinical details and previous H-scores by the first two pathologists. A final H score (0–300) was obtained by multiplying the intensity (1–3) and reactivity extension (0–100) for each case. Mean score was taken for the whole group of samples and samples with H scores above mean would be classified as of higher expression levels whereas those with H scores below mean would be classified as of lower expression levels.

2.6. Western blotting

Whole-cell lysates were prepared in 1× RIPA lysis buffer (Rock-land) with addition of 1% protease inhibitor cocktail (Sigma). Total protein (80 μg for *Akt* and 50 μg for other proteins) was fractionated using SDS-PAGE and thentransferred to nitrocellulose membranes (ExPro). After blocking with 5% non-fat milk in Tris buffered saline, membranes were incubated overnight at 4 °C with various primary antibodies and then probed with HRP-conjugated secondary antibodies (anti-rabbit or anti-mouse, Abcam) for 2 h at 4 °C. Primary antibodies to *LATS2*, *ERK 1/2*, *p-ERK1/2*, *Akt*, *p-Akt* (T308), *p-Akt* (S473) and *p53* were from Cell Signaling. β-actin (Sigma) was used as a loading control. Immune complexes were visualized by using ECL detection kit (GE Healthcare, Japan), and the band intensity was quantified by Image J software.

2.7. LATS2 siRNA transfection

Cells were transfected using the Hiperfect reagent according to the manufacturer’s instructions (Qiagen, UK). The target sequence of *LATS2* specific siRNA (siLATS2) is 5′-CTCCGCAAAGGGTACACTCAA-3′, and 5 nM siRNA was added to silence *LATS2*. One negative control siRNA (Qiagen, UK) was also included. Cultured cells were harvested for RNA or protein extraction at baseline and at 48 h.

2.8. Statistical analysis

Data were analyzed with SPSS 18.0 software. Differences between groups were estimated using the Chi-squared test, the Student’s *t*-test, or the log-rank test. Disease-free survival (DFS) and overall survival (OS) curves were calculated by the Kaplan–Meier method. Stepwise multiple regression models were built to determine the clinical parameters that independently predict either PFS or OS. Log rank tests were used to compare cumulative survival between different groups. All p values were two-sided and $p < 0.05$ was considered statistically significant. Cox proportional regression model was applied for multivariate analysis. A probability level of 0.05 was used to determine statistical significance.

3. Results

3.1. Demographics of lung cancer patients

Fifty patients were recruited prospectively before they underwent surgical resection, with 23 (46.9%) male and 26 (53.1%) female patients and with age range of 38–88 years (mean ± S.D., 64.1 ± 9.2 years). The ratio of non-smokers (61%) to ex- or current smokers (39%) was around 2 to 1. 28 tumors were *EGFR* wild-type (57%) with no mutation at exons 18–21 while 21 were *EGFR* mutants that showed at least one *EGFR* mutation in exons 18–21 (43%). The details can be found in Table 1.

3.2. LATS2 mRNA expression in lung AD patients

AD tissue samples expressing *LATS2* mRNA at levels above the mean expression level (6.3; range 0–13.1) were assigned to the high expression group (mean expression value 9.4, $n = 23$), and samples with expression less than the mean value were considered as the low expression group (mean expression value 3.6, $n = 26$).

LATS2 mRNA levels showed no significant differences between different clinical parameters, including gender, age, smoking history, pathological stage and *EGFR* mutation status (Table 1). Survival analysis indicated that high *LATS2* expression group had significantly longer disease-free survival (DFS) ($p = 0.002$, Fig. 1A) and overall survival (OS) ($p = 0.041$, Fig. 1B) than the low *LATS2* expression group. Furthermore, *LATS2* mRNA levels correlated with DFS (partial correlation ratio, 0.37; $p = 0.012$). Multivariate analysis further confirmed that *LATS2* mRNA level was a significant prognostic factor for survival status (DFS: hazard ratio, 0.221; $p = 0.003$; OS: hazard ratio, 0.238; $p = 0.036$) independent of age, gender, smoking history and staging (Table 2). Meanwhile, pathological stage (hazard ratio, 5.102; $p = 0.009$), as well as the presence of *EGFR* mutations (hazard ratio, 0.207; $p = 0.006$) were also significant prognostic factor of DFS (Table 2). When dividing samples based on both *EGFR* mutation status and *LATS2* level, patients with wild-type *EGFR* as well as expressing low *LATS2* expression displayed

3.3. LATS2 protein expression in lung AD tumors

Immunohistochemistry (IHC) staining on the independent set of 79 paraffin blocks of AD indicated that *LATS2* protein was expressed at relatively low levels in both the cytoplasm and the nucleus (Fig. 2C). After excluding 16 cases with zero H-score in both compartments, paired test revealed that *LATS2* protein expression was significantly higher in the cytoplasm than in the nucleus ($p < 0.001$). There was an association between *LATS2* IHC scores in the cytoplasm and gender with *EGFR* mutation status, in which female patients with *EGFR* mutations displayed modestly low levels of *LATS2* cytoplasm expression ($p = 0.031$). In males, *EGFR* wild-type cohort expressed slightly higher cytoplasmic staining of *LATS2*. These observations with *LATS2* IHC were consistent with the findings of *LATS2* expression at mRNA levels among gender with *EGFR* mutation status groups in a different set of tumor specimens with real-time PCR assay done as described in the previous section. Nuclear *LATS2* expression levels were generally low and no remarkable difference was found between all the clinical parameters examined.

3.4. LATS2 mRNA expression in AD cell lines

Before transfection, *LATS2* protein levels were quantified in each cell line (Fig. 2A). Basal *LATS2* expression in H2023, HKULC-2 and H1650 were much higher, while H1648, HKULC-4, H1975 and PC9 exhibited relatively lower expression levels of *LATS2*. After being transfected with *LATS2*-specific siRNA (siLATS2) or Negative Control siRNA (siCont), *LATS2* expression was successfully silenced in all cell lines confirmed with reduced levels of *LATS2* protein (Fig. 2B).

3.5. LATS2 modulates ERK pathway in the EGFR wild-type AD cell lines with high LATS2 expression

After successfully silencing *LATS2* expression in AD cell lines, we first examined for changes in phosphorylation of mitogen-activated protein kinase, *ERK1/2* (Fig. 3A). In most of the AD cell lines studied except for H1975 in which we failed to detect the activation of *ERK1/2*, the levels of phosphorylated *ERK1/2* did not alter significantly with *LATS2* silencing. Nevertheless, a modest increase in the phosphorylation status of *ERK1/2* was observed in H2023.

3.6. LATS2 knockdown differentially influences Akt activation in different lung AD cell lines

As another *EGFR*-activated signaling cascade, *Akt* pathway could also be modulated by *LATS2* kinase. siRNA silencing of *LATS2* increased levels of Thr308-phosphorylated *Akt* in H2023 (Fig. 3B). Since phosphorylation of *Akt* at Thr308 could enhance *Akt* activity, relatively high basal *LATS2* expression in this cell lines would inhibit *Akt* activation. Similar to the situation of *ERK* activity, *LATS2* knockdown did not affect *Akt* phosphorylation in neither HKULC-4 which expressed very low level of *LATS2* nor cell lines, PC9 and H1975, with *EGFR* mutations.

Phosphorylation at Ser473, which contributes to maximal *Akt* Activity, was also improved by *LATS2* knockdown in HKULC-2 cells (Fig. 3C). Together with above results, *LATS2* might diminish *Akt* activation in H2023 and HKULC-2cells. Both cell lines showed high *LATS2* expression at baseline. In H1650, the addition of *LATS2* siRNA slightly reduced the level of phosphorylated *Akt*at Ser473. Although the difference was not significant, it was still plausible that *LATS2* may differentially influence *Akt* activation and the regulation might be independent of *EGFR* mutation status.

3.7. Silencing of LATS2 induces p53 accumulation in lung AD cell lines with low basal p53 levels

Previous reports have indicated that *LATS2* is able to stabilize p53 thus facilitating p53-dependent checkpoint response to mitotic stress in breast cancer and osteosarcoma cells. However, in HKULC-2 cells, *LATS2* knockdown markedly increased total *p53* levels (Fig. 3D), suggesting that, instead of inducing *p53* accumulation, *LATS2* may actually downregulate*p53* protein expression in these cells. Noticeably, HKULC-2 cells expressed very low amount of p53 at baseline (Fig. 3D). Another two cell lines, HKULC-4 and H1650, which also exhibited low basal p53 expression, LATS2 silencing likewise enhanced p53 levels although the differences were not statistically significant.

4. Discussion

In this study, we demonstrated that low *LATS2* expression was an independent and significant predictor of poor overall survival. Furthermore, expression of *LATS2* correlated with DFS of these lung adenocarcinoma patients (partial correlation ratio, 0.37; $p = 0.012$). These findings suggested that *LATS2* may express tumor suppressive effects in lung adenocarcinoma.

In this study, patients with tumors harboring EGFR mutations exhibited longer median DFS than those with wild-type EGFR (median DFS: 33.4 vs. 27.2 months, respectively; $p = 0.006$). None of the recruited patients received TKI treatment. Other than complete resection for most of these early stage tumors, no treatment intervention was apparent. The influence of EGFR gene mutation on prognosis as shown in this study deserves further investigation. This unusual observation may stem from differences in ethnicity and pathological stage of recruited patients, as well as different detection methods of EGFR mutations, compared to reported data [27–29,30–32]. Since wild type *EGFR* also significantly predicted inferior DFS, it is not surprising that tumors with wild type *EGFR* and low *LATS2* expression exhibited the shortest DFS (overall $p = 0.004$, Fig. 1C). In non-smokers, in which *EGFR* mutations are more common, significant difference in DFS was still observed between two *LATS2* expression groups ($p = 0.010$, Fig. 1D). Similar observation has been made before, that knockdown of YAP (Yes kinase-associated protein), whose functions can be inhibited by *LATS2*, sensitizes cancer cells to EGFR-TKI erlotinib [33]. Further studies are warranted to investigate the relationships between *LATS2* expression levels and therapeutic responses of these patients.

Immunohistochemistry suggested that AD tumors expressed *LATS2* in both cytoplasm and nucleus. During interphase, *LATS2* mainly remained in the cytoplasm, especially localized to the centrosome [8]. After phosphorylated by *Aurora A* kinase during mitosis, *LATS2* could translocate to the chromosome and the central spindle, thus modulating chromosome segregation and cytokinesis [9,10,34]. To date, the best characterized function of *LATS2* is the regulation of *YAP*/*TAZ* proto-proteins via canonical Hippo signaling mainly in breast, colon and hepatic cancers as well as in malignant pleural mesothelioma [15,35,36] but not lung adenocarcinomas. *LATS2* might still plays critical roles in non-canonical Hippo signaling and even in Hippo independent pathways, such as the G-protein-coupled receptor (GPCR) signaling [37,38] and the *K-ras*/*Raf-1* axis [39,40]. The exact roles, as well as the relationship between the subcellular localization and the biological functions of *LATS2*, in lung adenocarcinomas need further exploration.

Our data suggested that *LATS2* can suppress *ERK* signaling in *EGFR* wild-type lung adenocarcinoma cell lines with high basal *LATS2* expression. Another study conducted in HeLa cells identified three down-regulated genes in siRNA-*LATS1/2*-treated cells, namely *SPRED1*, *SPRY2* and *SPRY4* [41]. These three proteins act as negative regulators of the pathway [42–45], indicating that *LATS2* might inhibit *ERK* activation through regulating some members of the Sprouty proteins. Additionally, as an upstream activator of *ERK1/2*, any change in *Ras* protein levels after silencing of *LATS2* may contribute to observed alterations in *ERK1/2* activities. However, in si*LATS2*-treated H2023, no detectable change in *Ras* protein expression was observed (Fig. 4). This implies that *LATS2* regulate *ERK* phosphorylation via other mechanisms in H2023. It is mentionable that, in most of lung AD cell lines studied *LATS2* knockdown induced *Ras* protein expression (Fig. 4). The consequence of this modulated *Ras* expression by *LATS2* needs further investigation.

The regulation of *LATS2* on *Akt* signaling was more complicated and independent of *EGFR* mutation status. In two *EGFR* wild-type cell lines (H2023 and HKULC-2), *LATS2* could reduce *Akt* activation by inhibiting *Akt* phosphorylation at either Thr308 or Ser473. This alternation may probably result from the ability of *LATS2* to maintain the protein phosphatase-2A catalytic subunit (PP2A-C) level [39] which in turn leads to dephosphorylation of *Akt* Thr308 [46]. On the other hand, the interaction between *LATS2* and *Akt* may be reciprocal and linked by mammalian sterile 20-like kinase-1 (*Mst1*). Apart from an upstream activator of *LATS2*, *Mst1* has been reported to function as an inhibitor of *Akt* [47], and, meanwhile, *Akt* signaling leads to phosphorylation of *Mst1* at Thr120 and limits *Mst1*-mediated tumor suppressive functions [48,49]. The role of *LATS2* in this network deserves further study. However, *LATS2* may also positively modulate *Akt* activity since silencing of *LATS2* in H1650 triggered a mild decrease in the levels of phosphorylated *Akt*. As H1650 exhibits loss of *PTEN* gene [50] and constitutive activation of *Akt*, downregulation of *LATS2*in this cell line, which may in turn inhibit *Akt* activity, is possible to compensate this abnormal signaling transduction.

In this study, *LATS2* may suppress *p53* expression, which is contradictory to previous studies that *LATS2* can cause *p53* induction in response to mitotic apparatus damage [7] as well as oncogenic activation [51]. That may be partially explained by the absence of mitotic or oncogenic stress introduced to cells in this study. On the other hand, in NSCLC tumors, downregulated *LATS2* mRNA levels have been found to be highly correlated with lower expression of *Mdm2* which leads to *p53* ubiquitination and degradation [17,18], implying that diminished *LATS2* expression might positively regulate *p53* protein level, as seen in HKULC-2, HKULC-4 and H1650. All of three cell lines express very low amount of *p53* at baseline, while two of them, HKULC-2 and H1650, show relatively high basal *LATS2* levels. So it is reasonable to deduce that the low basal *p53* expression may partially result from the corresponding high basal expression of *LATS2*. Additionally, based on two studies that *Snail* has been found to suppress *p53* in A549 lung carcinoma cell line [52] and *LATS2* can act as a positive regulator of Snail1 protein [53], we could speculate that *LATS2* may negatively mediate *p53* activity through *Snail*. Further research can include these two candidates, *MDM2* and *Snail*, to disclose the comprehensive interaction between *LATS2* and *p53* in lung adenocarcinoma cells. Moreover, since silencing of *LATS2* would induce *p53* accumulation, we might postulate that *LATS2* is linked to drug sensitivity through regulation of *p53*-mediated processes [54].

In conclusion, relatively higher level of LATS2 gene correlates with the better survival in patients with stage resected lung AD. *In vitroLATS2* knockdown suggested that this differential expression could impact *EGFR*-activated signaling network, particularly in high LATS2 expression group, and *p53* pathway. LATS2 also appeared to modulate different signaling pathways via phosphorylated ERK or Akt in EGFR wild-type and EGFR mutant lung AD cell lines respectively. Thus, *LATS2* gene expression in lung AD patients warrant further investigations for being a potential biomarker for survival status in lung cancer patients of both early and advanced stages as well as the role of LATS2 expression in modulating different signaling pathways in either EGFR mutant or EGFR wild-type lung cancer.

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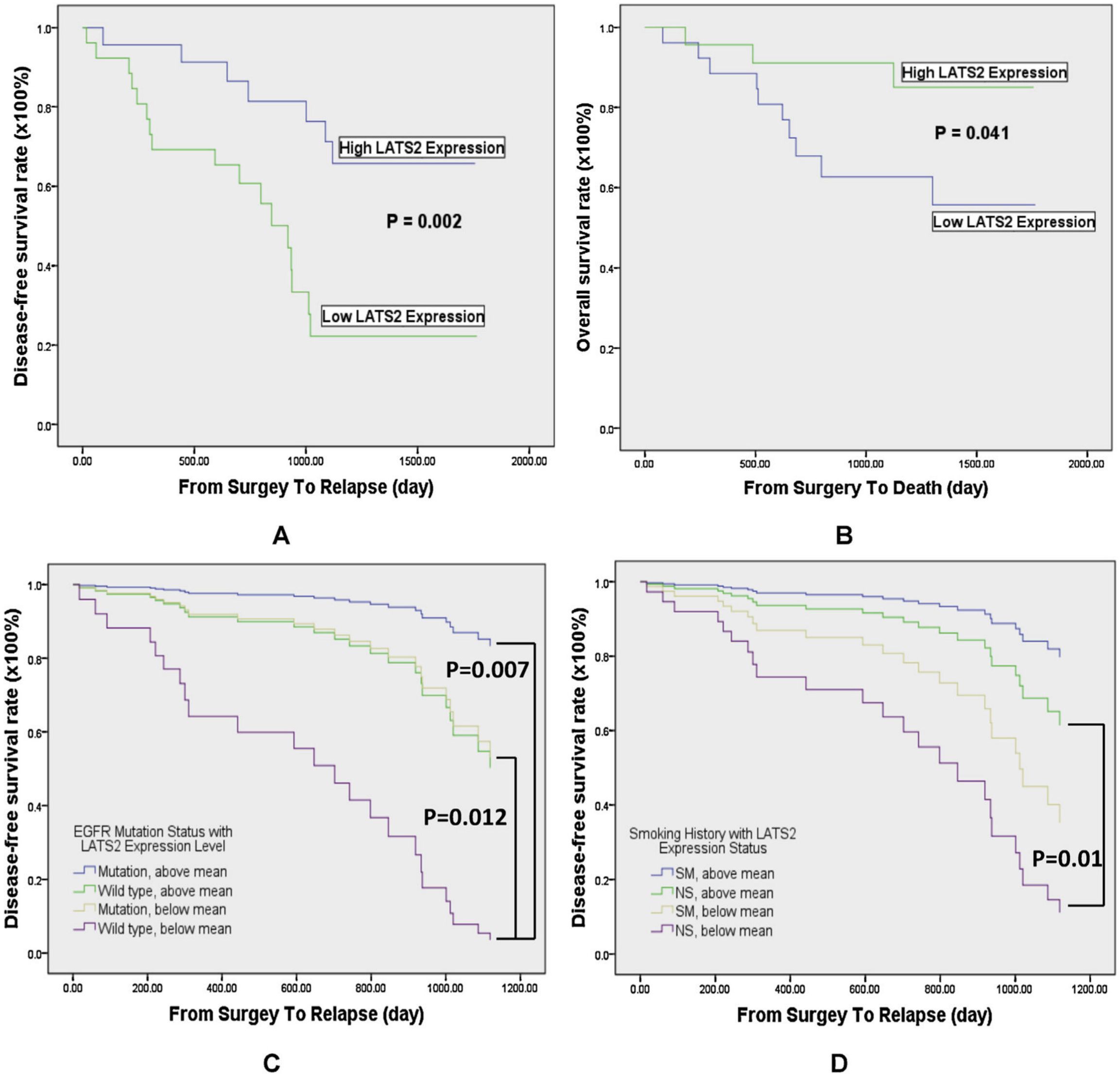
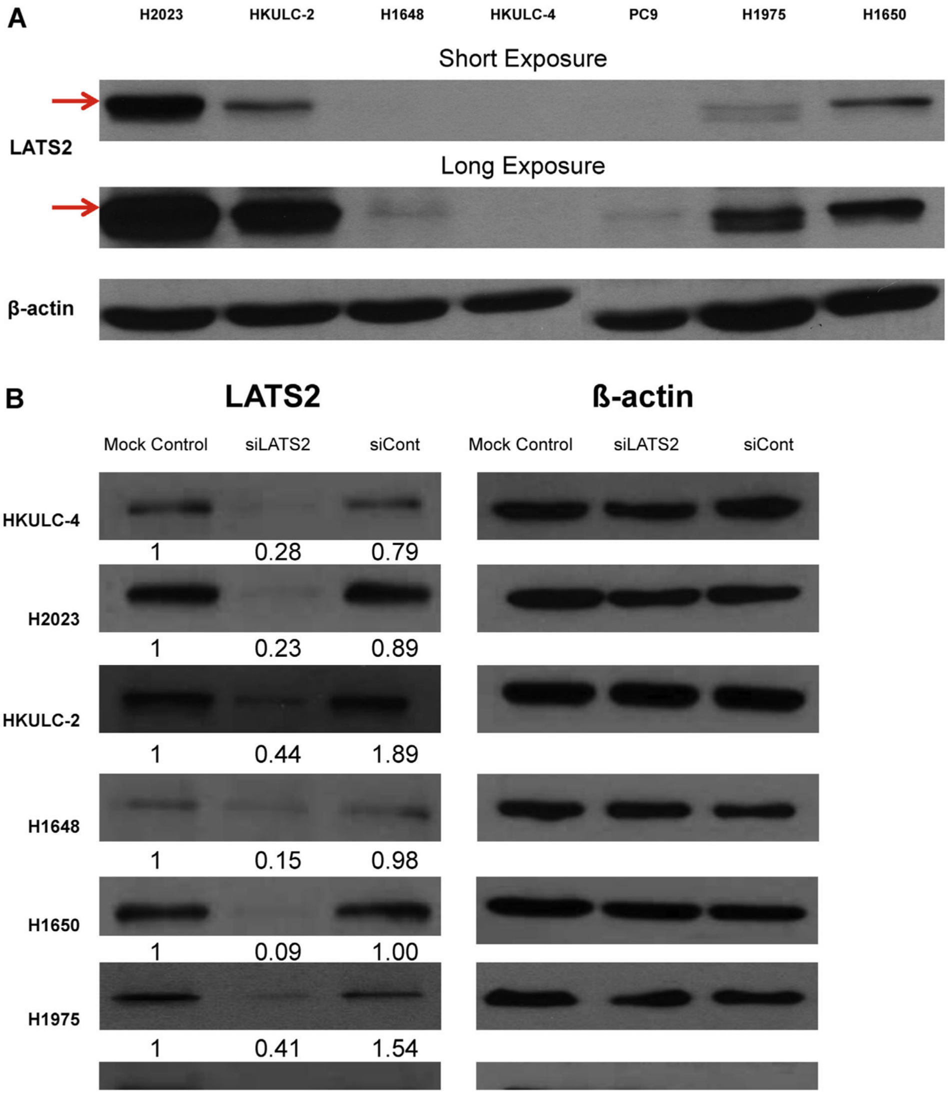


Fig. 1.

Survival analysis of disease-free survival (DFS) and overall survival (OS). (A) Significant difference in disease-free survival between high and low LATS2 expression groups was present. The p-value was estimated by the log-rank test. (B) AD patients with high LATS2 expression had a significantly better overall survival than those expressing low LATS2. (C) Cox regression analysis demonstrated that patients in the wild type EGFR together with low LATS2 expression group exhibited the worst DFS. (D) In non-smoker patients, high LATS2 expression is a significant predictor of better DFS. poor DFS (Fig. 1C). Inferior DFS was also observed in non-smokers exhibiting low *LATS2* levels (Fig. 1D).


A
H2023
HKULC-2
H1648
HKULC-4
PC9
H1975
H1650
Short Exposure
LATS2
Long Exposure
β-actin
B
LATS2
ß-actin
Mock Control
siLATS2
siCont
Mock Control
siLATS2
siCont
HKULC-4
1
0.28
0.79
H2023
1
0.23
0.89
HKULC-2
1
0.44
1.89
H1648
1
0.15
0.98
H1650
1
0.09
1.00
H1975
1
0.41
1.54

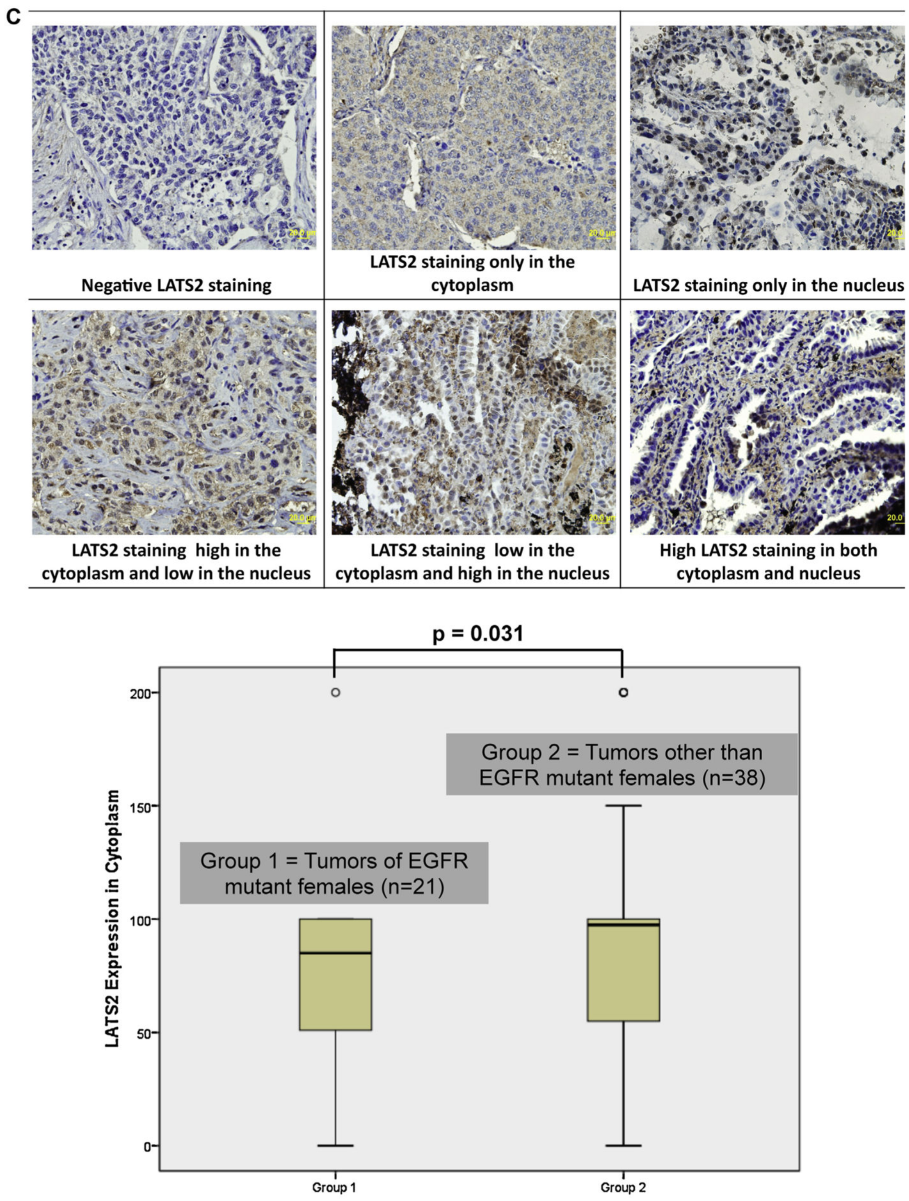
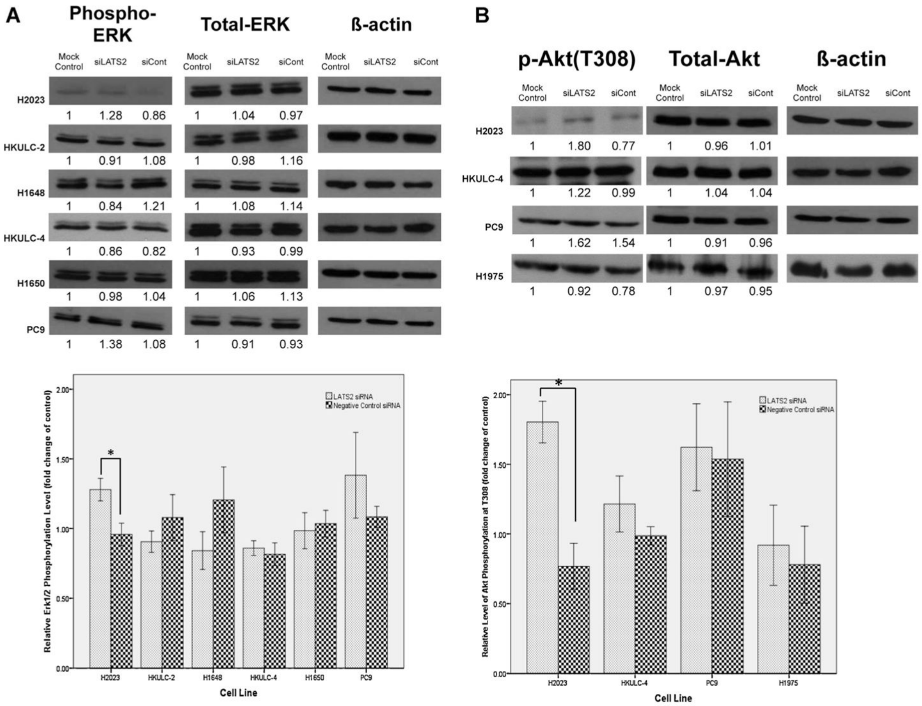


Fig. 2.

LATS2 protein expression in AD tumors and cell lines. (A) Before transfection, LATS2 protein expression was measured in each cell lines. (B) Cells were transfected with siRNA for LATS2 (siLATS2), Negative Control siRNA (siCont) or none (Mock Control) for 48hr. Number below each blot indicates fold change to mock control (n = 3). (C) Immunohistochemistry staining of LATS2 expression in clinical samples of AD. Female cases bearing EGFR mutations had moderately lower expression of LATS2 in the cytoplasm (mean expression value, 77.7 vs. 92.5, p = 0.031).


A
Phospho-ERK
Total-ERK
ß-actin
Mock Control
siLATS2
siCont
H2023
1 1.28 0.86
1 1.04 0.97
HKULC-2
1 0.91 1.08
1 0.98 1.16
H1648
1 0.84 1.21
1 1.08 1.14
HKULC-4
1 0.86 0.82
1 0.93 0.99
H1650
1 0.98 1.04
1 1.06 1.13
PC9
1 1.38 1.08
1 0.91 0.93
Relative Erk1/2 Phosphorylation Level (fold change of control)
2.00
1.50
1.00
0.50
0.00
*
LATS2 siRNA
Negative Control siRNA
H2023
HKULC-2
H1648
HKULC-4
H1650
PC9
Cell Line
B
p-Akt(T308)
Total-Akt
ß-actin
Mock Control
siLATS2
siCont
H2023
1 1.80 0.77
1 0.96 1.01
HKULC-4
1 1.22 0.99
1 1.04 1.04
PC9
1 1.62 1.54
1 0.91 0.96
H1975
1 0.92 0.78
1 0.97 0.95
Relative Level of Akt Phosphorylation at T308 (fold change of control)
2.00
1.50
1.00
0.50
0.00
*
LATS2 siRNA
Negative Control siRNA
H2023
HKULC-4
PC9
H1975
Cell Line

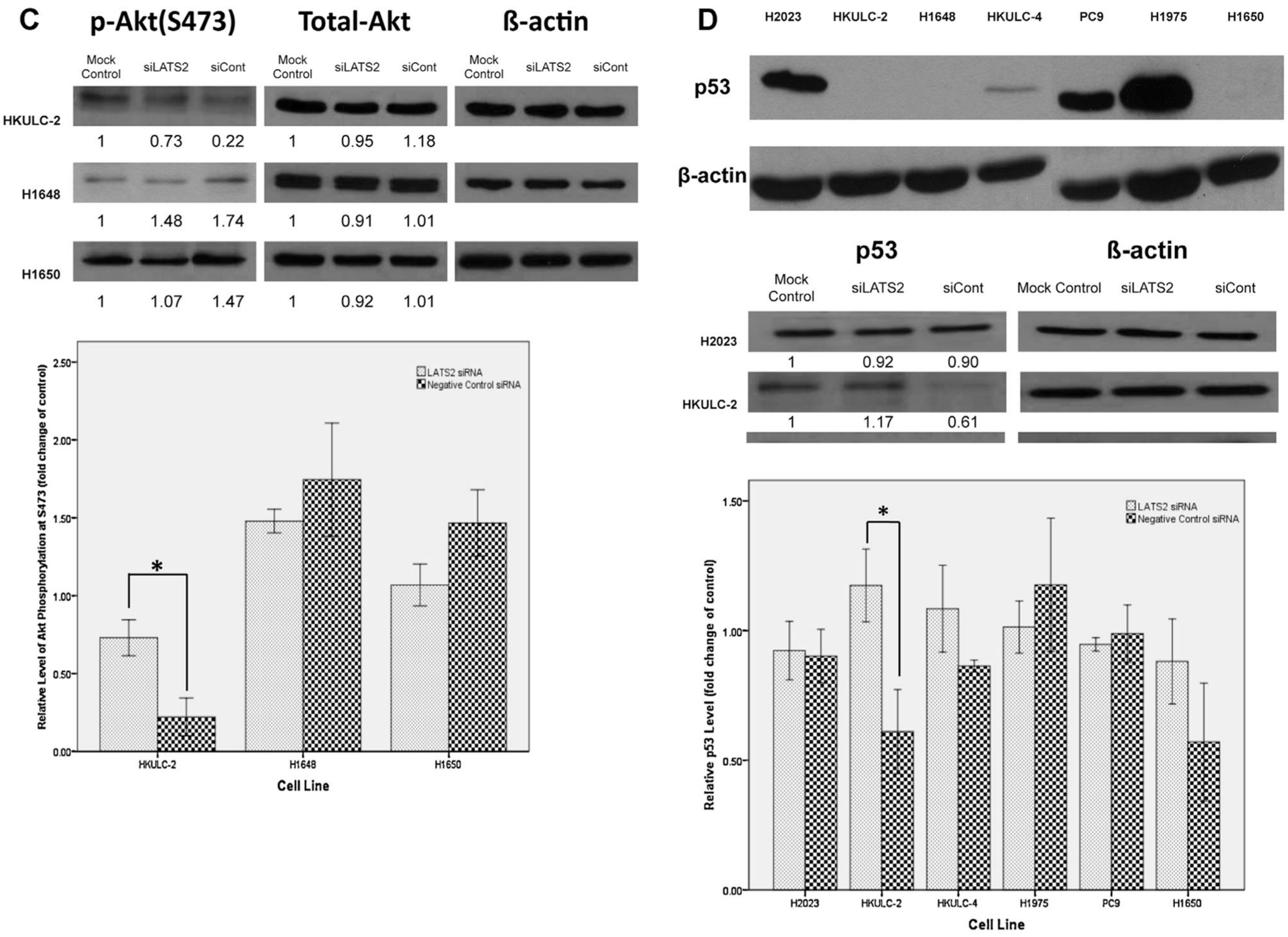


Fig. 3.

The effects of LATS2 silencing on ERK activation, Aktphosphorylation and p53 level. Cells were transfected with siRNA for LATS2 (siLATS2), Negative Control siRNA (siCont) or none (Mock Control) for 48 h. Cellular lysates were analyzed by Western blotting with the indicated antibodies. Number below each blot indicates fold change to mock control ($n = 3$). One asterisk indicates $p < 0.05$ determined by Student's *t*-test. (A) Changes in phosphorylated ERK levels in cell lines after 48-h transfection with siRNAs described above. (B) Analysis of Thr308-phosphorylated Akt levels in cells after transfected with siRNAs described above. (C) Levels of Ser473-phosphorylated Akt in HKULC-2, H1648 and H1650 cells with or without LATS2 knockdown. D) The top left picture showed the basal levels of p53 in different cell lines. Modulation of p53 levels in these cells after transfected with siRNAs described above was demonstrated in the lower left picture and in the graph.

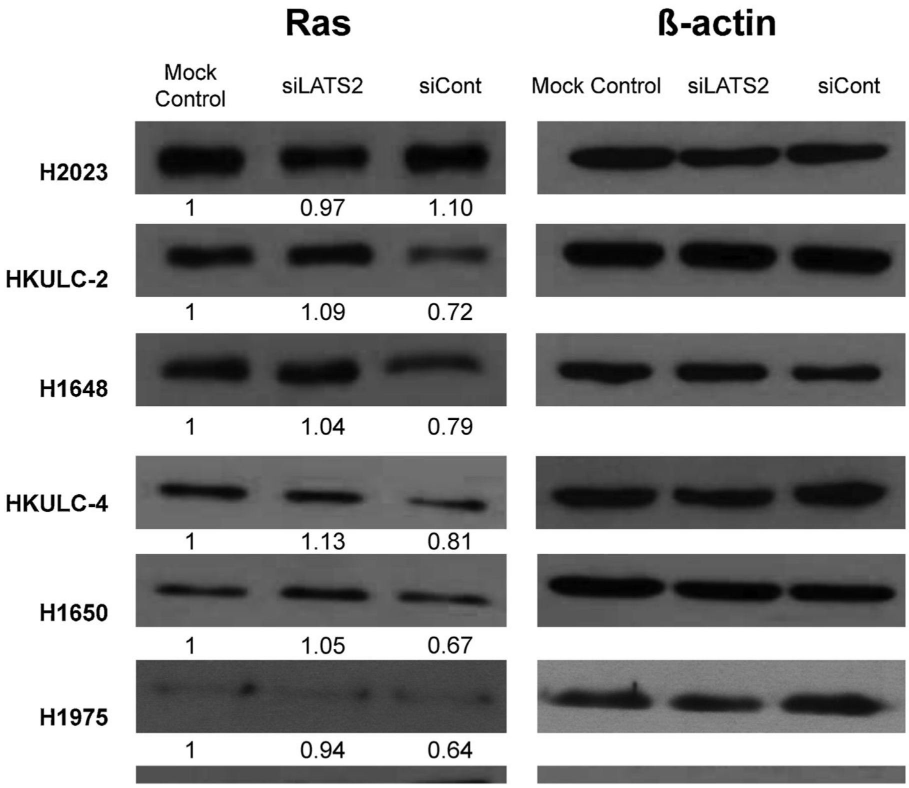


Fig. 4.

Modulation of Ras expression by LATS2. (A) Cells were transfected with siRNA for LATS2 (siLATS2), Negative Control siRNA (siCont) or none (Mock Control) for 48 h. Cellular lysates were analyzed by Western blotting with the indicated antibodies. Number below each blot indicates fold change to mock control ($n = 3$).

Table 1

Patient and tumor characteristics according to *LATS2* mRNA expression (A) and *LATS2* protein expression (B).

(A) *LATS2* mRNA expression

	High LATS2 expression	**Low LATS2 expression**	***P*-value**
N (%)	23 (46.9)	26 (53.1)	
Follow-up duration (day), mean ± S.D.	1276 ± 404		
Gender, *n* (%), M:F	23:26		0.907
Male	11 (22.4)	12 (24.5)	
Female	12 (24.5)	14 (28.6)	
Age, mean ± S.D.	64.1 ± 9.2		0.336
	66.3 ± 7.6	62.2 ± 10.2	
Smoking, *n* (%), SM:NS	19:30		0.590
SM	8 (16.3)	11 (22.4)	
NS	15 (30.6)	15 (30.6)	
Stage, *n* (%), I:II:III	29:12:08		0.709
Stage I	15 (30.6)	14 (28.6)	
Stage II	5 (10.2)	7 (14.3)	
Stage III	3 (6.1)	5 (10.2)	
In groups			0.706
Earlier stage (I and II)	20 (40.8)	21 (42.9)	
Advanced stage (III)	3 (6.1)	5 (10.2)	
Relapse, *n* (%)			0.015*
Yes	7 (14.3)	17 (34.7)	
No	16 (32.7)	9 (18.4)	
DFS (day), median	919		
	1303	746	0.006*
Death, *n* (%)			0.044*
Yes	3 (6.1)	10 (20.4)	
No	20 (40.8)	16 (32.7)	
OS (day), median	1095		
	1364	794	0.059
EGFR, *n* (%), Mut:WT	21:28		0.283
Mutation (Mut)	8 (16.3)	13 (26.5)	
Wild type (WT)	15 (30.6)	13 (26.5)	

(B) *LATS2* IHC staining

	Above mean	**Below mean**	***P*-value**
N (%)	32 (50.8)	31 (49.2)	
Gender, *n* (%), M:F	36:27		0.021*
Male	18 (36)	18 (36)	
Female	13 (26)	14 (28)	

(B) *LATS2* IHC staining

	Above mean	**Below mean**	***P*-value**
EGFR, *n* (%), Mut:WT	37:22		0.01[*]
Mutation (Mut)	18 (36)	19 (38)	
Wild type (WT)	11 (22)	11 (22)	

(C) *EGFR* mutation status of samples included in this study

		Samples for mRNA measurement	**Samples for IHC**
EGFR wild-type		28	22
	Exon 18	4	3
	Exon 19	8	10
	Exon 20	1	1
EGFR mutation	Exon 21	7	6
	L858R + T790M	1	1
	Unknown	0	27

[*] Statistically significant ($p < 0.05$).

Table 2

Multivariate analysis of disease-free survival (DFS) and overall survival (OS) (Cox proportional regression model).

	DFS			**OS**		
	HR	**95% CI**	***p*-Value**	**HR**	**95% CI**	***p*-Value**
Univariate analysis						
Gender (female/male)	1.129	0.497–2.566	0.772	2.364	0.769–7.270	0.133
Age	0.951	0.905–1.000	0.051	1.020	0.956–1.088	0.546
Smoking history (SM/NS)	1.157	0.477–2.807	0.747	0.561	0.187–1.678	0.301
Stage (I – II/III)	3.975	1.569–10.071	0.004*	1.040	0.229–4.719	0.960
EGFR mutation Status (WT/Mut)	0.577	0.247–1.351	0.205	0.348	0.095–1.268	0.110
LATS2 expression (low/high)	0.269	0.110–0.662	0.004*	0.281	0.077–1.029	0.055
Multivariate analysis						
Gender (female/male)	2.431	0.654–9.039	0.185	2.246	0.448–11.251	0.325
Age	0.997	0.939–1.058	0.913	1.015	0.933–1.103	0.729
Smoking history (SM/NS)	2.119	0.564–7.961	0.266	1.482	0.327–6.724	0.610
Stage (I – II/III)	5.102	1.512–17.208	0.009*	1.832	0.320–10.504	0.497
EGFR mutation Status (WT/Mut)	0.207	0.068–0.631	0.006*	0.304	0.071–1.306	0.109
LATS2 expression (low/high)	0.221	0.081–0.605	0.003*	0.238	0.062–0.908	0.036*

HR, hazard ratio; CI, confidence interval; SM, smoker; NS, non-smoker; WT, wild type; Mut, mutant.

[*] Statistically significant.