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DARS2 promotes the occurrence of lung adenocarcinoma via the ERK/c-Myc signaling pathway

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Abstract

Background: DARS2 expression is upregulated in lung adenocarcinoma (LUAD) which correlates with tumor patient stage and prognosis. The mechanism of DARS2 involvement in LUAD still needs to be further explored.

Methods: In this study, we found that DARS2 expression in LUAD tissue was significantly higher than that in normal tissue. At the same time, the Kaplan–Meier curve showed that the survival prognosis of LUAD patients with high expression of DARS2 was significantly worse than low expression of DARS2. The expression of DARS2 was detected in LUAD and adjacent normal tissues by IHC staining, histochemical scoring and a survival curve was generated. In addition, we demonstrated that the knockdown and overexpression of DARS2 significantly affected the proliferation, invasion, and migration of LUAD cells in vitro and in vivo. Finally, western blot and rescue assay were performed on LUAD cells to further explore and verify the signaling pathway.

Results: DARS2 expression was significantly upregulated in LUAD tissues and cell lines. What is more, the increased expression of DARS2 was closely related to proliferation, invasion and metastasis. The tumorigenic assay in nude mice further showed that the tumorigenic ability of nude mice was significantly improved with the increase in DARS2 expression. Finally, we determined that DARS2 plays its role in LUAD by targeting the ERK/c-Myc signaling pathway.

Conclusion: Our data revealed the oncogenic role of DARS2 in LUAD, indicating that DARS2 may be a predictive biomarker and novel therapeutic target for LUAD.

KEYWORDS
DARS2, ERK/c-Myc signaling pathway, lung adenocarcinoma

INTRODUCTION

Cancer is the main cause of death worldwide. It is now widely believed that cancer is a complex disease composed of multiple histological and molecular types.[1] Lung adenocarcinoma (LUAD), as a major malignant tumor, is one of the most common cancer-related deaths in the world,[2] despite great efforts being made to improve early detection and develop optimal treatment strategies.[3] In terms of treatment strategy, patients with LUAD still face the problems of high postoperative recurrence rate and poor survival rate. Therefore, it is necessary to find new molecular targets to improve the treatment of LUAD and predict the prognosis of LUAD patients.[4,5]

Aminoacyl tRNA synthetases (ARSs) are an important class of enzymes with an evolutionarily conserved protein synthesis machinery.[6] ARSs are essential enzymes covalently linking substrate amino acids to cognate tRNAs for protein synthesis and ARSs also function as regulators of cellular processes by sensing different cellular conditions.[7] In this process, each ARS interacts with an amino acid and an ATP molecule to produce a high-energy aminoacyl adenylate (aa-AMP) intermediate and a pyrophosphate (PPi) molecule. Following this, the intermediate binds to the cognate tRNA and transfers the amino acid to it. Ultimately, the aminoacylated tRNA is recruited to the ribosome to participate in protein synthesis.[8,9]

Tao Fang and Jin Jiang contributed equally to this work.

In fact, there are 36 ARSs in a typical human cell, which can function in the cytoplasm or mitochondria.[10] These molecules are involved in a variety of physiological and pathological processes, including cysteine polysulfation, angiogenesis, post-translational modifications, immune response and neurological development.[8,10–12] More notably, there is growing evidence that ARSs are quite closely associated with tumor development. For example, the oncogene IARS2 promotes non-small cell lung cancer tumorigenesis by activating the AKT/MTOR pathway;[13] EARS2 significantly coexpresses with PALB2 in breast and pancreatic cancers.[14] Of interest to us is DARS2, a gene encoding a protein belonging to the class II amido-tRNA synthetase family. Mutations in this gene have been associated with white matter encephalopathy, brainstem and spinal cord involvement, and elevated lactate (LBSL).[15,16] In addition, HBV upregulation of DARS2 has been reported to promote hepatocarcinogenesis via the miR-30e-5p/MAPK/NFAT5 pathway.[17] However, the mechanism of action of DARS2 in LUAD is unclear.

In our study, we found that DARS2 was significantly overexpressed in LUAD patients, and the overexpression of DARS2 was closely related to the poor prognosis and pathological stage of LUAD patients. In addition, we validated the carcinogenic effect of DARS2 through in vitro and in vivo experiments. We found that high expression of DARS2 can promote the proliferation, invasion and metastasis of LUAD in vitro. Subsequently, a nude mice tumor experiment further validated the cancer oncogenic effect of DARS2, and the relevant indicators DARS2 and Ki-67 were validated through immunohistochemistry experiments. After that, we further discussed the mechanism of DARS2 promoting proliferation, invasion and metastasis of the lung adenocarcinoma cells. Finally, our experiment demonstrated that DARS2 can target the ERK/c-Myc signaling pathway and further validate it by adding ERK inhibitors.

In conclusion, our study confirmed that targeting DARS2 to treat LUAD is a feasible strategy and provides a potential new target for the diagnosis and treatment of LUAD patients.

METHODS

Bioinformatic analysis and clinical samples

We analyzed the expression of DARS2 in a variety of cancers through the TIMER2.0 database (http://timer.comp-genomics.org/) to complete the screening of primary molecular markers. In addition, the LUAD data in TCGA (The Cancer Genome Atlas) were analyzed through the UALCAN online database (http://ualcan.path.uab.edu.). Expression analysis, prognostic analysis and pathological staging analysis were included.

The tissue microarray of LUAD tissues (HLugA180Su08) was obtained from Shanghai Outdo Biotech Company. The microarray contained 84 LUAD tissues and 81 adjacent normal tissues including the survival information of each patient. The Ethics Committee of Shanghai Outdo Biotech Company approved this study (no. SHYJS-CP-1904014).

Cell cultures and transfection

LUAD cell lines H1299, A549 and PC9 were obtained from Shanghai Academy of Sciences. The medium used was PPMI-1640 plus 10% fetal bovine serum (FBS; Gibco) and cells were incubated at 37°C with 5% CO_2. The siRNA for DARS2 mRNA was produced by GenePharma. The siRNA template sequences in our study were applied as following: siDARS2, sense CACCUAUGGAACUGAUAAATT; antisense UUUAUCAGUUCCAUAGGUGTT. According to the manufacturer's protocol, the H1299 cells and A549 cells were transfected with siRNA using the jetPRIME transfection reagent (Polyplus-transfection, Illkirch). Both H1299 and PC9 cells were transfected with lentivirus, and the tumor cells with stable corresponding expression were finally obtained after drug selection.

Western blot

Proteins in cells or tissues were hydrolyzed with 1% protease and phosphatase inhibitor cocktail (Beyotime Biotechnology). The extracted protein was then dissolved with 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. The next day, after washing three times with TBST, they were incubated with secondary antibodies for 1 h at room temperature. Then, the signal was detected through the manufacturer's recommended enhanced chemiluminescence. The antibodies used were as follows: DARS2, Proteintech (13807-1-AP); GAPDH, ZSJQ-BIO (TA-08); Ki-67, HuaBio (ET1609-34); E-cadherin, CST (#14472); N-cadherin, CST (#13116); vimentin, HuaBio (ET1610-39); ERK, HuaBio (SA43-03); p-ERK, HuaBio (ET1603-22); c-Myc, HuaBio (RT1149).

Cell counting kit-8 assay

Microplates from 96 wells were implanted for a number of 2000 (H1299, A549 and PC9 cells) per well. Each well was treated with a 10% CCK-8 solution for 2 h. The cells were cultured for 24, 48, 72, and 96 h, and were then recorded at 450 nm per well with a microplate reader.

5-ethynyl-2′-deoxyuridine (EdU) assay

The transduced cells were inoculated into 96-well plates under 8000–10 000 cell/well conditions. EdU staining was carried out with an EdU staining kit (RiboBio) following the manufacturer's instruction with 2 h of EdU incubation. EdU imaging was performed using fluorescence microscopy (Olympus).

Transwell assay

Migration studies were performed using a 24-well plate across the well chamber (BD Biosciences, #353092). About 2×10^4 LUAD cells cultured in different conditions were implanted in the upper chamber and suspended in 200 μL of serum-free medium, and the lower chamber was filled with 800 μL of 1640 containing 20% FBS. After 24 h of incubation at 37°C, the cells were removed from the upper surface of the membrane with a cotton swab. Migrating cells in the lower half of the filter were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 60 min at room temperature. The number of cells migrating to the lower surface was counted in three randomly selected high-magnification fields for each sample.

Wound healing

For wound healing, we cultured a suitable proportion of cells in six-well plates. The principle of overnight inoculation was 100% concentrated. The cell layer at the bottom of the well was scratched with a sterile 200 μL pipette tip, creating a linear gap. The plates were photographed under inverted fluorescence microscopes for 24 h, respectively. The healing rate of the open wound was calculated. The scratch healing rate = (healing width at 24 or 36 h − healing width at 0 h)/ healing width at 0 h.

Immunohistochemistry

LUAD tissues and nude mice tumors were fixed with 4% paraformaldehyde at room temperature for 60 min. After being incubated at 60°C for 1 h, dewaxed, and rehydrated, antigen extraction was carried out with citrate buffer (pH 6) at 97°C for 20 min. A solution of 3% hydrogen peroxide was applied to block endogenous peroxidase activity for 10 min at room temperature. Nonspecific binding of antibodies was avoided by incubating the slides with 5% normal goat serum in TBST for 2 h at room temperature. The slides were then incubated with the primary antibodies against Ki67 and DARS2 at 4°C overnight. After washing three times with TBST, each slide was incubated with the appropriate HRP-labeled secondary antibody, and the signal was developed with DAB solution before counterstaining with hematoxylin. The slides were then captured using an inverted microscope.

In vivo experiments

Weitonglihua Animal Company (Beijing, China) provided the 4-week-old BALB/c nude mice. Before the experiment, five healthy nude mice were randomly divided into two groups; the experimental and control groups and were placed in two cages. A subcutaneous tumor formation experiment was carried out after 1 week of careful feeding. We observed the nude mice and the subcutaneous tumors closely every day before the experiment was terminated on the 25th day. The nude mice used in the experiment were euthanized, and the subcutaneous tumors were stripped out, weighed and photographed. The tumors were then completely immersed in 4% paraformaldehyde solution and preserved. The Medical Ethics Committee of Shandong University's Qilu Hospital approved and supervised all the experiments.

ERK inhibitors

Order specific ERK1/2 phosphorylation inhibitor SCH772984 (2.5 mM, HY-50846, MedChemExpress, China), was prepared and dissolved in dimethyl sulfoxide (DMSO) and frozen in a refrigerator at −80°C. We stabilized the overexpression of DARS2 in PC9 cells and control cells one day in advance. The inhibitor SCH772984 was added to one vector and one DARS2 gene overexpression well and an equal volume of DMSO was added to the other vector and one DARS2 gene overexpression well, and incubated for 24 h. After 24 h, the inhibitory effect of SCH772984 was analyzed by western blot and subsequent CCK-8 response experiments were conducted.

Genetic alteration analysis

Using SangerBox 3.0 (http://www.sangerbox.com/tool), a useful online bioinformatic tool (the "pan cancer analysis mRNA modification" module), mutation samples were detected in the TCGA-LUAD cohort, with a total of 445 (86.7%) plot samples. We used chi-square tests to evaluate the differences in gene mutation frequency in each group of samples.

Statistical analysis

Differences between the two groups were analyzed using *t* tests in GraphPad Prism 8, in which *t* tests were used to compare differences between the two groups. Log rank tests were used to analyze the differences between the survival curves. And p-value < 0.05 was considered statistically significant.

RESULTS

DARS2 expression elevated in LUAD tissues and cell lines and associated with poor prognosis

Our research gained preliminary insights from the expression of the DARS2 gene in cancer through rigorous statistical analysis in the TCGA database. We finally identified that

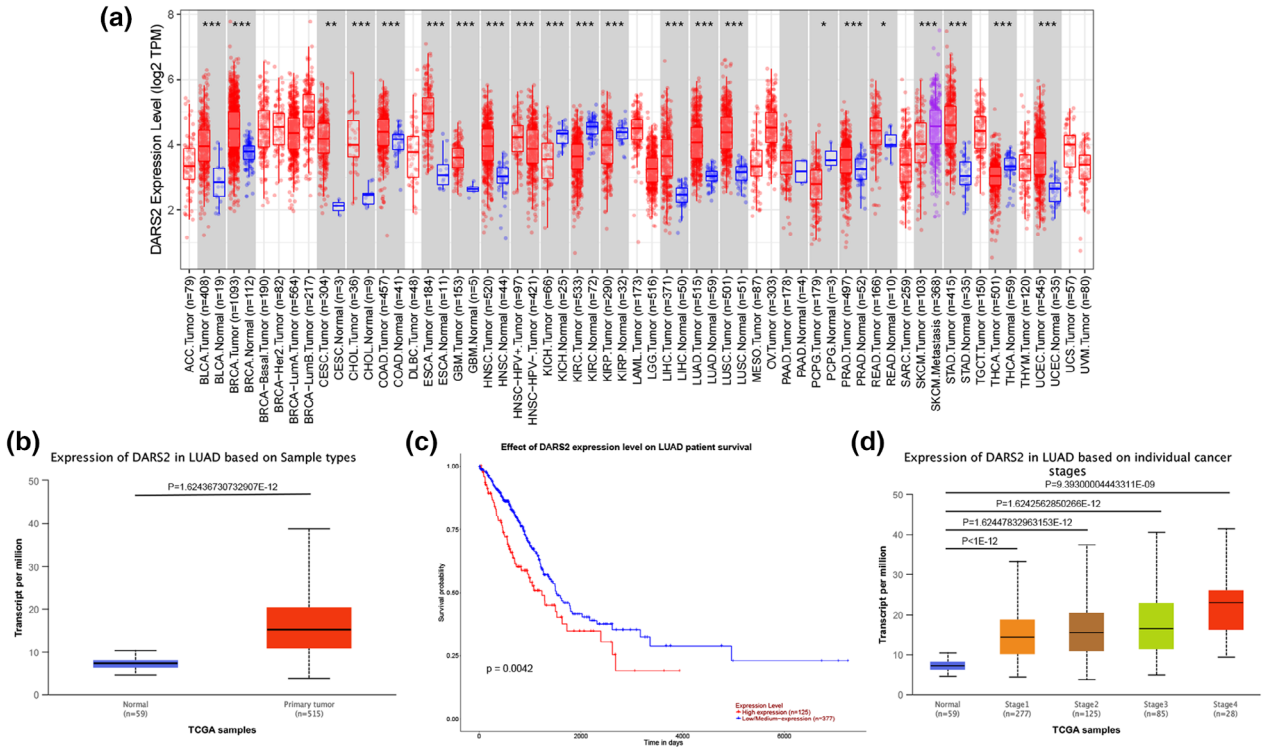

FIGURE 1 Bioinformatic analysis of DARS2 expression. (a) DARS2 expression was shown to be elevated in a variety of cancers in the Timer 2.0 database. (b) The expression of DARS2 was upregulated in lung adenocarcinoma (LUAD) patients. (c) The Kaplan–Meier analysis showed that the overall survival (OS) of LUAD patients with higher expression of DARS2 had reduced significantly. (d) DARS2 is closely associated with the stage of LUAD patients.

DARS2 mRNA expression was significantly upregulated in different species of cancers (Figure 1a). On the UALCAN website, expression analysis revealed that 515 LUAD cases as well as 59 normal cases were statistically analyzed in the TCGA database, which concluded that DARS2 was significantly highly expressed in LUAD cases ($p = 1.62436730732907E-12$) (Figure 1b). Meanwhile, we statistically analyzed 125 LUAD patients with high DARS2 expression as well as 377 with low DARS2 expression in the TCGA database and found that patients with high DARS2 expression were always followed by a significantly worse prognosis than those with low DARS2 expression ($p = 0.0042$) (Figure 1c). We further analyzed the relationship between normal cases and LUAD cases at different stages through the ULCAN website, which showed that DARS2 expression was closely related to the stage of LUAD (Figure 1d).

At the next stage, we further analyzed the expression of DARS2 in LUAD by immunohistochemical assays (IHC) in order to verify the expression of DARS2 more accurately. By performing IHC experiments on 84 LUAD tissues as well as 81 normal paraneoplastic tissues, we came to an accurate conclusion that the main location of DARS2 expression was in the cytoplasm and that DARS2 was highly expressed in LUAD, as well as high expression of DARS2 was closely associated with poor prognosis of LUAD (Figure 2a–e). Simultaneously, we continued to explore the expression of DARS2 in normal lung epithelial cells and LUAD cells, and examined the expression of DARS2 in normal lung epithelial cells as well as LUAD cells by western blot assay. The results showed that DARS2 was significantly more expressed in LUAD cell lines (H1299, A549 and PC9) than in human normal lung epithelial cells (BEAS-2B) (Figure 2f).

DARS2 promotes proliferation of LUAD cells

To further investigate the possible impact of DARS2 in the development of LUAD, we selected small interfering RNA that specifically target the DARS2 gene and transfected them in H1299 as well as A549 cell lines, resulting in decreased expression of DARS2 in each cell line. Moreover, transfection of overexpression virus in PC9 cells resulted in upregulation of DARS2 expression in LUAD (Figure 3a–c). Next, we performed experiments to verify the effect of DARS2 on cell proliferation. The CCK-8 assay results showed that the proliferative capacity of H1299 and A549 cell lines was significantly reduced after transfection with siDARS2 compared to siNC. Furthermore, the results showed that PC9 cells transfected with DARS2-OE became more proliferative and had increased cell survival compared to the vector group. In conclusion, our CCK-8 assay showed that high expression of DARS2 promoted the proliferative capacity of LUAD cell lines (Figure 3d–f).

And EdU assay was also used to verify the effect of DARS2 on the proliferative capacity of LUAD cell lines. The results showed that the proportion of proliferating cells in

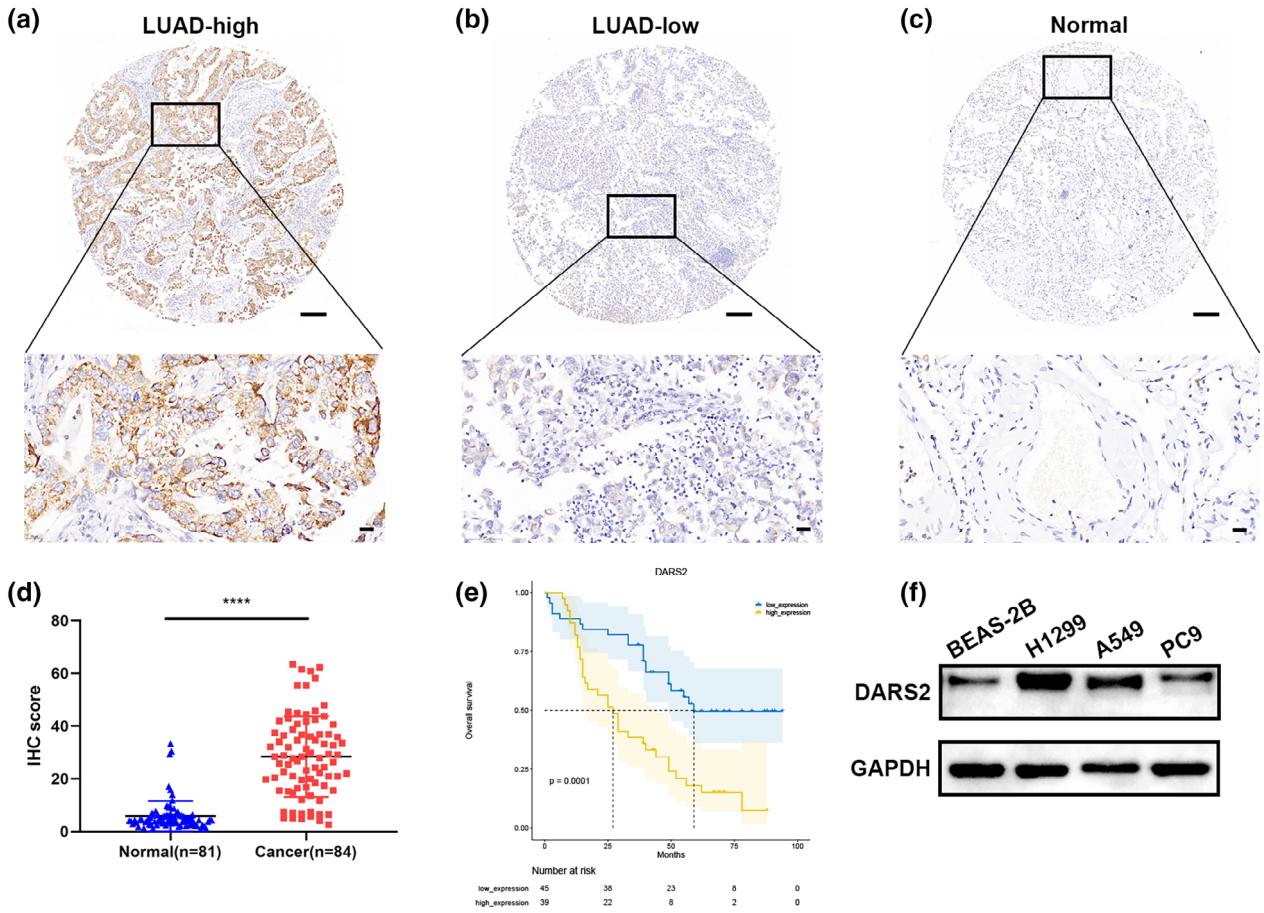

FIGURE 2 DARS2 was highly expressed in lung adenocarcinoma (LUAD) tissues and cells. (a) Representative immunohistochemical (IHC) staining images of high expression of DARS2 in LUAD. (b) Representative IHC images of low expression of DARS2 in LUAD. (c) Representative IHC images of DARS2 expression in normal lung tissues. (d) IHC scores of DARS2 in tissue microarrays of LUAD. (e) Survival analyses of the LUAD patients based on tissue microarrays via Kaplan–Meier analysis. (f) Western blotting was used to detect the expression of DARS2 in BEAS-2B, H1299, A549, and PC9 cells.

H1299 as well as A549 cell lines transfected with siDARS2 was significantly lower compared to siNC. In contrast, the proportion of proliferating cells in PC9 cell lines transfected with DARS2-OE was significantly higher compared to the vector group (Figure 3g–i). It was again verified that high expression of DARS2 could promote the proliferative capacity of LUAD cell lines.

DARS2 promotes invasive metastasis of LUAD cells

To explore whether DARS2 affects the ability of LUAD cells to invade and metastasize, we performed transwell and wound healing assays. Compared to the siNC group, A549 and H1299 cells transfected with siDARS2 had significantly fewer cells crossing the membrane into the lower chamber, indicating that these LUAD cells were less able to invade and metastasize. However, PC9 cells transfected with DARS2-OE had a statistically significant increase in cells crossing the membrane into the lower chamber compared to the vector group. This suggested that the increased expression of DARS2 promotes the capability of invasive transfer (Figure 4a–c). Moreover, we further verified the followed results by wound healing assays. A549 and H1299 cells transfected with siDARS2 had a slower migration rate compared to the siNC group, indicating that the invasive metastatic ability of A549 and H1299 cells was reduced with the knockdown of DARS2. The results of PC9 cells transfected with DARS2-OE were significantly higher than the vector group (Figure 4d–f).

DARS2 promotes tumorigenic capacity of LUAD cells in vivo

We acquired lentiviruses which are capable of stably knocking down DARS2 by siRNA sequences. The H1299 cells transfected with the DARS2 knockdown lentivirus were amplified to produce sufficient quantities of sh-DARS2 and sh-NC (Figure 5a). The two groups of cells were then injected subcutaneously into each of the two groups of nude mice. The tumors were carefully examined on a daily basis, and their length and width observed daily once they had

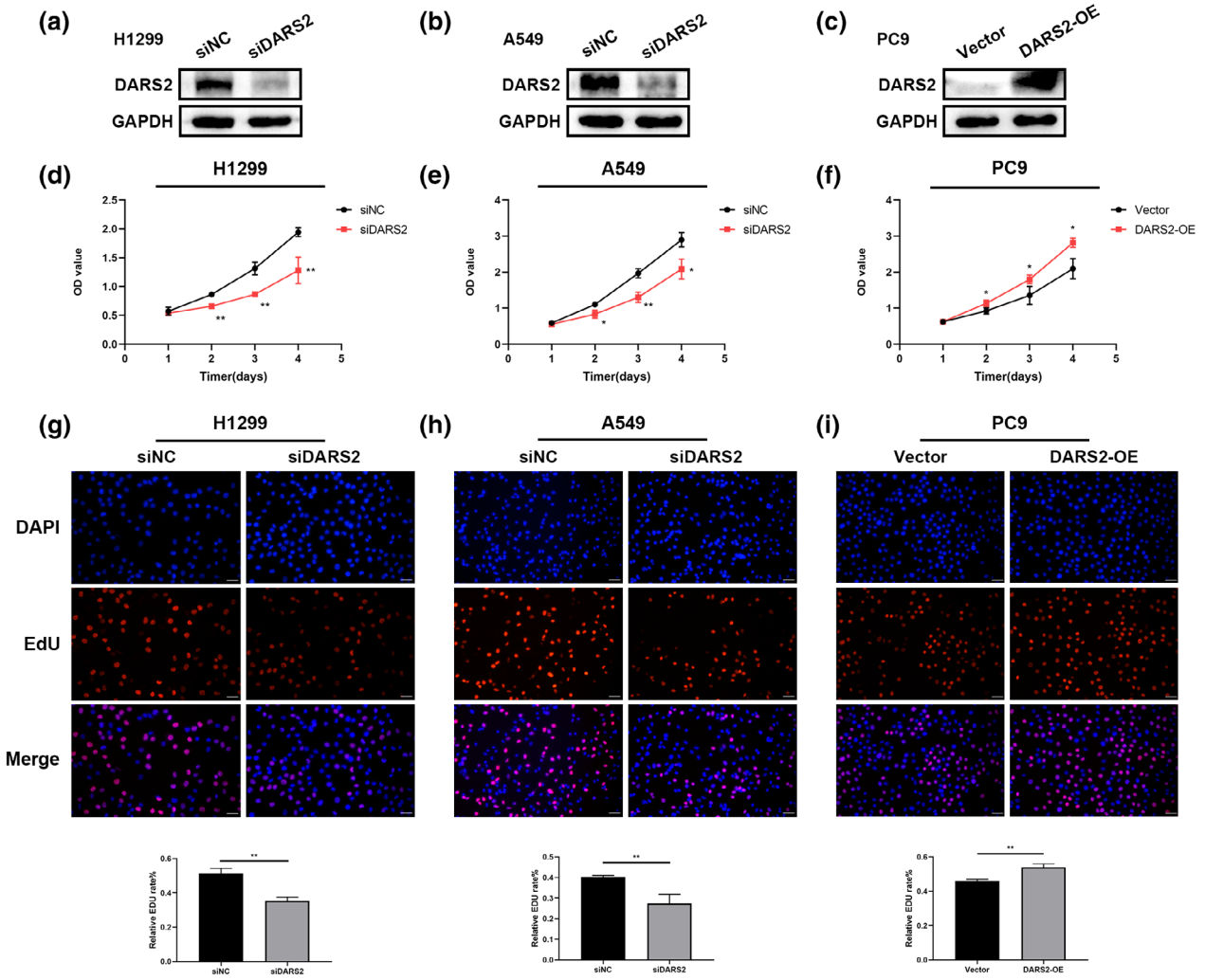

FIGURE 3 Highly expressed DARS2 promotes proliferation of LUAD cells in vitro. (a–c) Western blot assay was performed to detect the efficiency of DARS2 knockdown and overexpression in H1299, A549 and PC9 cells. (d–f) The proliferative ability of cells was detected by CCK-8 assay. The knockdown of DARS2 resulted in diminished cell proliferation capacity in H1299 and A549, while it was opposite in PC9 cell lines that overexpressed DARS2. Data are expressed as mean ± standard deviation. (g–i) Cell proliferation ability was detected by 5-ethynyl-2′-deoxyuridine (EdU) assay. The results showed that knockdown of DARS2 resulted in diminished cell proliferation capacity in H1299 and A549 cells, while it was opposite in the PC9 cell lines where DARS2 was overexpressed. Data are expressed as mean ± standard deviation. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

appeared at the injection site. When the tumors were large enough, they were removed and photographed for our records. The results suggested that the tumor volumes in the sh-DARS2 group were relatively small compared to those in the sh-NC group and were statistically significant (Figure 5b,c). IHC experiments subsequently verified that the expression of DARS2 as well as Ki-67 was significantly lower in the sh-DARS2 group than in the sh-NC group (Figure 5d). Simultaneously, we selected PC9 cell lines for transfection with the DARS2 overexpressing lentivirus DARS2-OE and vector group (Figure 5e). The tumor volume of the DARS2-OE group was relatively larger than that of the vector group when inoculated in nude mice using the same method, which was also statistically significant (Figure 5f,g). These results suggests that DARS2 promoted the tumorigenic ability of LUAD cells in vivo. Meanwhile, IHC experiments showed the expression of DARS2 as well as Ki-67 was significantly higher in the DARS2-OE group than in the vector group (Figure 5h). Thus, we further verified that DARS2 could promote the development of LUAD through in vivo experiments.

DARS2 participate in LUAD progression via ERK/c-Myc signaling pathway

To investigate the signaling pathways affected by DARS2, we reviewed the literature source and found that studies have reported that upregulated DARS2 affects the MAPK pathway in hepatocellular carcinoma. Therefore, we further validated the targeting pathway of DARS2 by western blot. We found that when DARS2 was knocked down in H1299 and A549 cell lines, p-ERK1/2 and c-Myc expression were downregulated while ERK1/2 remained largely unchanged

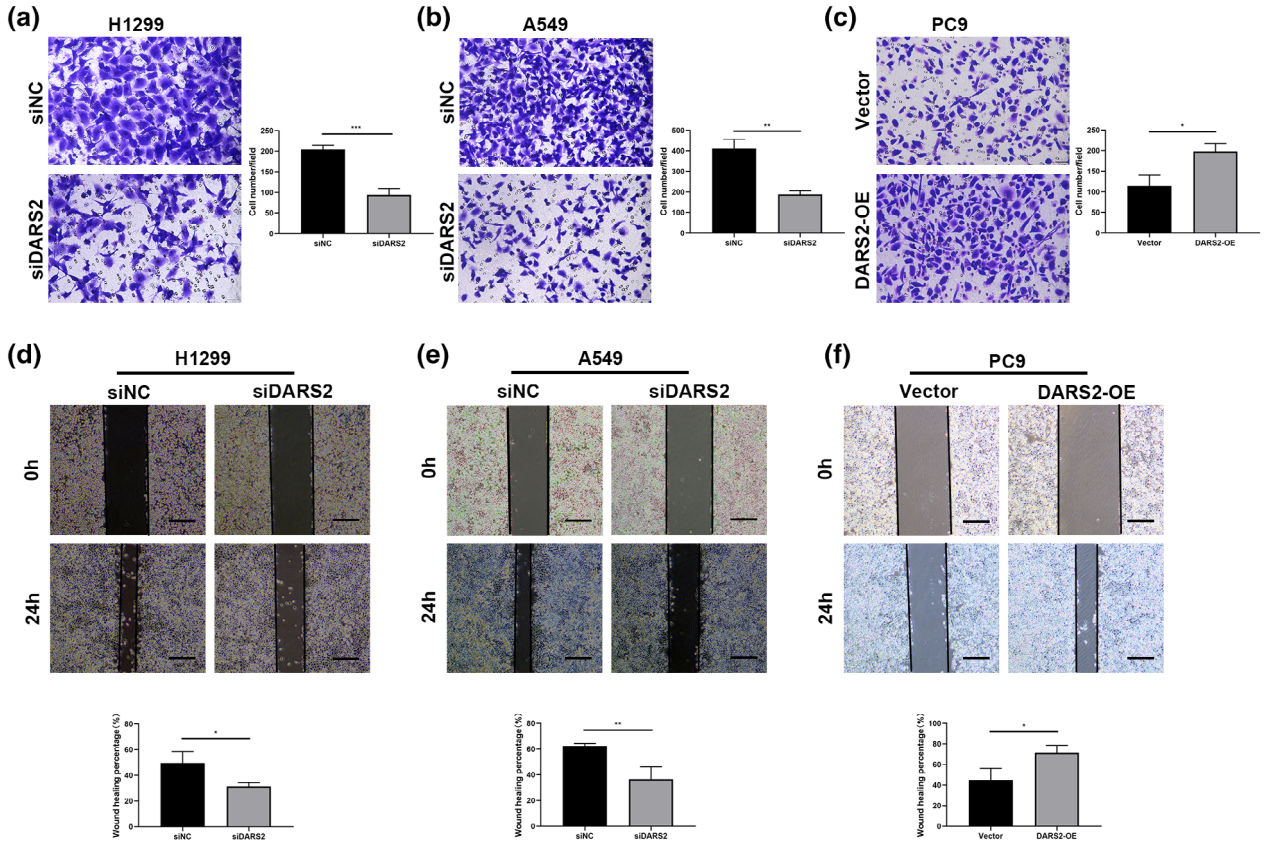

FIGURE 4 Highly expressed DARS2 promotes invasive and metastasis of lung adenocarcinoma (LUAD) cells in vitro. (a–c) Transwell assay revealed that the knockdown of DARS2 in H1299 and A549 cells resulted in the decrease of invasive and metastasis, while it was opposite in the PC9 cell line where DARS2 was overexpressed. (d–f) Wound healing assay revealed that the knockdown of DARS2 in H1299 and A549 cells resulted in the decrease of invasive and metastasis, while it was opposite in the PC9 cell line where DARS2 was overexpressed. Data are expressed as mean ± standard deviation. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

(Figure 6a,b). Meanwhile, the opposite effect was obtained when DARS2 was overexpressed in PC9 cells (Figure 6c). Furthermore, to address this feature of tumor invasion and metastasis, we also examined markers related to epithelial-mesenchymal-transition (EMT). Western blot showed that when DARS2 was knocked down, the mesenchymal markers of H1299 and A549 (N-cadherin, vimentin) decreased and epithelial markers (E-cadherin) increased when DARS2 was knocked down (Figure 6d,e). The opposite result was observed after overexpression of DARS2 in PC9 cells (Figure 6f).

Rescue experiments and genetic alteration analysis of DARS2 in LUAD

To further demonstrate that DARS2 promotes tumor progression through activation of the ERK/c-Myc pathway in LUAD cells, we performed rescue experiments by using the ERK-specific inhibitor SCH772984 in PC9 cell lines overexpressing DARS2.[18] Through experiments, SCH772984 specifically inhibited the phosphorylation of p-ERK1/2 and c-Myc pathways, which can be reversed by DARS2 overexpression. (Figure 7a). Furthermore, the CCK-8 assay results showed that the proliferation of LUAD cells in the vector and DARS2-OE groups was significantly weaker than that in the group without drug addition, confirming that the ERK inhibitor SCH772984 was able to attenuate the increased proliferation of cells in PC9 cells due to DARS2 overexpression (Figure 7b). In conclusion, our experiments demonstrate that DARS2 was able to perform a functional role by affecting the ERK/c-Myc pathway. Furthermore, we identified 15 mutated genes in the mutation profile of the DARS2 high/low expression queue in the TCGA-LUAD queue through the Sangerbox 3.0 database. The top five genes were *TP53*, *TTN*, *CSMD3*, *ZFHX4* and *KRAS* (Figure 7c).

DISCUSSION

LUAD is the leading subtype of lung cancer and is a common cause of cancer-related death worldwide.[19] The treatment of LUAD has advanced development within multiple strategies including chemotherapy, surgery, radiotherapy, immunotherapy, targeted therapy and combination therapy.[20,21] However,

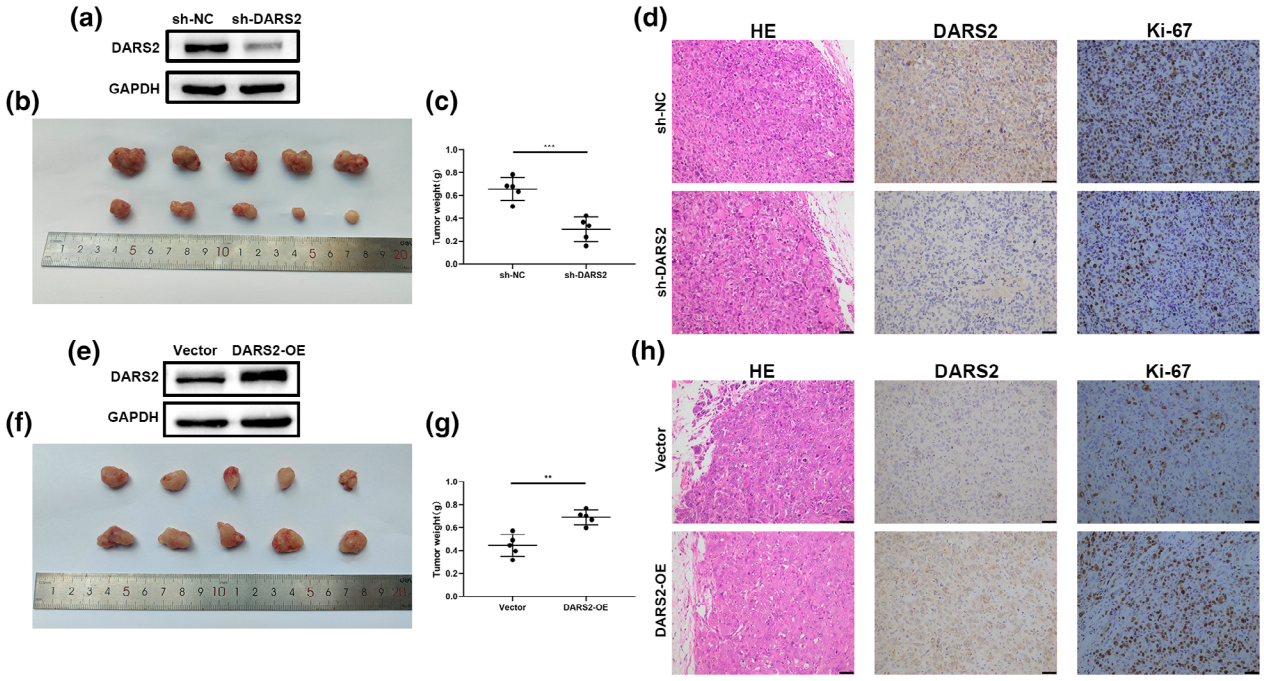

FIGURE 5 High expression of DARS2 promotes tumorigenesis in nude mice. (a–c) The subcutaneous tumors of nude mice in the sh-DARS2 group were much smaller tumors and weighed less in comparison with the sh-NC group. (d) Immunohistochemistry (IHC) and hematoxylin-eosin (HE) staining subsequently verified that the expression of DARS2 as well as Ki-67 was significantly lower in the sh-DARS2 group than in the sh-NC group. (e–g) Compared with the vector group, the subcutaneous tumors of nude mice in the DARS2-OE group were larger and weighed more than the vector group. (h) IHC and HE staining were used to detect the expression of Ki-67 and DARS2 in the subcutaneous tumors of nude mice. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

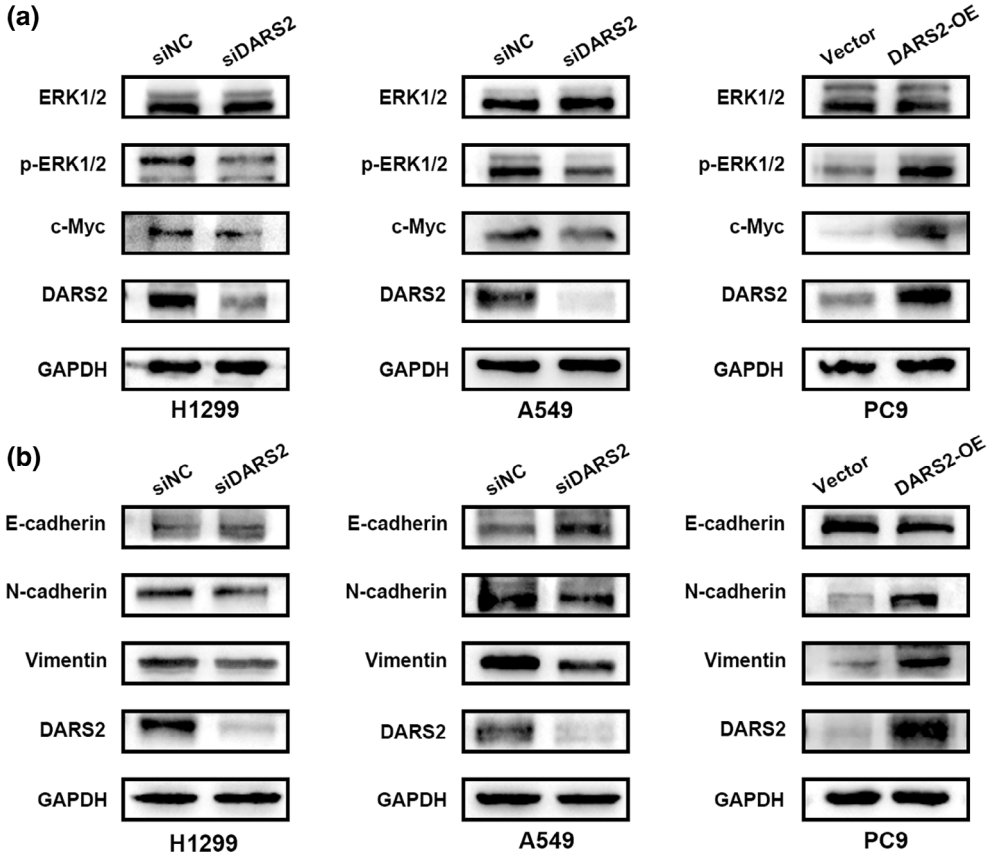

FIGURE 6 DARS2 exerts oncological effects through the ERK/c-Myc pathway in lung adenocarcinoma (LUAD). (a) The expression of ERK/c-Myc signaling pathway was detected using western blot (WB). (b) WB was used to detect the epithelial-mesenchymal-transition (EMT) biomarkers.

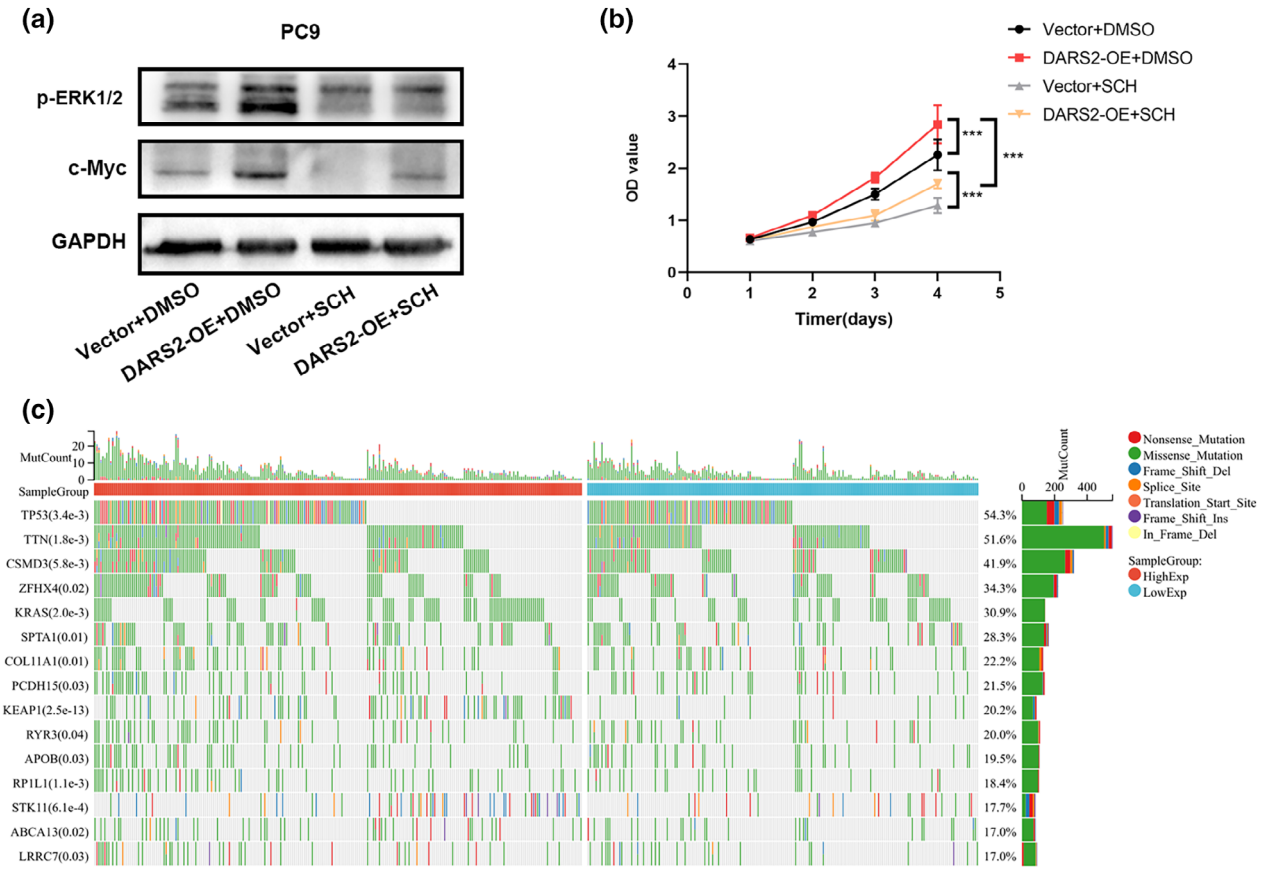

FIGURE 7 Rescue experiments and genetic alteration analysis of DARS2 in lung adenocarcinoma (LUAD). (a) DARS2-OE PC9 cells were treated with the specific ERK1/2 phosphorylation inhibitor SCH772984 or the same volume of dimethyl sulfoxide (DMSO) for 24 h. The expression of the ERK/c-Myc signaling pathway was detected by western blotting. (b) Cell counting kit-8 (CCK-8) assay was used to detect the ERK/c-Myc signaling pathway after the addition of ERK1/2 phosphorylation inhibitor for 24 h to stabilize the proliferative capacity of DARS2 overexpressing PC9 cells. (c) The top 15 mutation genes ranked in the DARS2 high and low expression queue in LUAD. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$).

due to the complexity and heterogeneity of tumorigenesis, the overall survival (OS) of patients with LUAD remains relatively low. Therefore, it is crucial that new molecular targets to improve the treatment for LUAD and predict patient prognosis are found.

Aminoacyl-tRNA synthetases are an important class of enzymes that are recruited to the ribosome and have an evolutionarily conserved mechanism for protein synthesis.[22] ARSs can function in the cytoplasm or mitochondria and are involved in a variety of physiological and pathological processes.[23] DARS2 is a mitochondrial enzyme that specifically aminoacylates aspartyl-tRNA.[24] The gene is located on chromosome 1, has 17 exons and is capable of encoding a mitochondrial aspartyl-tRNA synthetase.[25] In current research, it has been found that DARS2 is quite closely associated with the development of cancer. For example, DARS2 markers are valuable in the differential diagnosis of malignant mesothelioma (MM).[26] Upregulation of DARS2 by HBV promotes hepatocarcinogenesis through the miR-30e-5p/MAPK/NFAT5 pathway.[17] However, the mechanism of action of DARS2 in LUAD has been rarely reported and requires further discussion.

In our study, we determined through bioinformatic analysis and immunohistochemistry experiments that DARS2 plays an important role in LUAD, providing a reliable foundation for our future studies. Next, considering that tumor cells are aberrant in their ability to proliferate, we observed changes in the proliferation of LUAD tumor cells by knocking down as well as overexpression of the DARS2 gene. As expected, we observed that high expression of DARS2 promoted the proliferative capacity of LUAD cells through CCK-8 and EdU assays. In addition, another important characteristic of known tumor cells is invasive metastasis. We performed wound healing and transwell assays to verify this feature. We found that the invasive metastatic ability of H1299 and A549 cells was significantly reduced after knocking down DARS2. However, the invasive metastatic ability of PC9 cells was significantly enhanced by overexpression of DARS2. Our results demonstrate that DARS2 can promote the development and progression of LUAD. After that, we used a subcutaneous tumor formation assay in nude mice to objectively evaluate the effect of DARS2 on the proliferative capacity of LUAD. The results showed that the tumorigenic ability of nude mice with H1299 cells in the knockdown DARS2 group was significantly reduced compared to the NC group, in contrast to nude mice with PC9 cells in the overexpression DARS2 group, which had a significantly

enhanced tumorigenic ability. After removing and sectioning the tumors, we demonstrated that DARS2 promotes tumorigenic capacity in nude mice by performing IHC assays on DARS2 as well as Ki-67 protein, further validating the procarcinogenic role played by DARS2 in LUAD.

EMT is the process by which epithelial cells acquire mesenchymal characteristics.[27] The spread of cancer cells to distant organs is a major clinical challenge in cancer therapy. EMT is a key regulator of metastasis in some cancers by conferring an aggressive phenotype.[28] In our study, we showed that mesenchymal markers (N-cadherin, vimentin) were reduced and epithelial markers (E-cadherin) were increased in H1299 and A549 when DARS2 was knocked down. The opposite result was observed after overexpression of DARS2 in PC9 cells. These results were consistent with phenotypic experiments, which confirmed that DARS2 promotes the EMT process in LUAD cells.

The family of MAPK kinases, including ERK1/2, JNK and p38, responds to various extracellular stimuli and plays an important role in cell proliferation and invasive metastasis.[29,30] Available studies have shown that DARS2 promotes hepatocarcinogenesis in hepatocellular carcinoma through the miR-30e-5p/MAPK/NFAT5 pathway and is involved in the ERK/c-Myc pathway.[17] Extensive studies have demonstrated the important role of ERK1/2 signaling in lung gland cancers.[31] In addition, c-Myc had been reported to be an ERK substrate and is involved in a variety of oncogenic processes.[30,32] It is unclear whether DARS2 promotes the progression of LUAD through the ERK/c-Myc axis. Our results showed that when DARS2 was knocked down in H1299 and A549 cells, p-ERK1/2, c-Myc expression was downregulated and ERK1/2 was largely unchanged. Meanwhile, when DARS2 was overexpressed in PC9 cells, we obtained the opposite result. Thus, inhibition of DARS2 may lead to inhibition of the ERK/c-Myc pathway, thereby reducing cell proliferation in human LUAD. On this basis, we further validated our conclusions using ERK inhibitors.

Previous studies have reported that the ERK/c-Myc axis is closely related to mutations in cancer. Meanwhile, we found that DARS2 plays a role through the ERK/c-Myc signaling pathway in LUAD. Thus, we further explored the relationship between DARS2 expression and mutation in lung adenocarcinoma. In our study, we identified the top 15 genes with the highest mutation frequency in the DARS2 high/low expression queue. Among the 15 mutated genes, the *KRAS* gene was the one that interests us the most. *KRAS* accounts for 85% of RAS mutations observed in human cancers. At the same time, *KRAS* is present in 35% of lung adenocarcinomas (LUADs).[33] Research has proven ERK phosphorylation as a marker of RAS activity and its prognostic value in non-small cell lung cancer.[34] Hyperactivation of ERK by multiple mechanisms is toxic to RTK-RAS mutation-driven lung adenocarcinoma cells.[35] This may provide us with a new direction for studying the relationship between DARS2 and *KRAS* mutations in lung adenocarcinoma. However, there are still shortcomings in our study. First, we need to further conduct in vivo experiments to distinguish the effects of overexpression in different actual treatments. Second, the relationship between DARA2 and mutations in lung adenocarcinoma needs further discussion.

In summary, the DARS2 expression is significantly elevated in LUAD patients and cell lines, which correlates with stage and poor prognosis. At the same time, DARS2 promotes proliferation, survival and invasive metastasis of LUAD cells and promotes the rate of tumorigenesis of LUAD cells. Moreover, DARS2 targets ERK/c-Myc signal pathway to promote the occurrence and development of LUAD.

AUTHOR CONTRIBUTIONS

Study conception and design: Tao Fang and Jin Jiang. Administrative support: Hui Tian. Collection and assembly of data: Jin Jiang and Wenhao Yu. Data analysis and interpretation: Tao Fang and Rongyang Li. Manuscript writing: Tao Fang and Jin Jiang.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to disclose on the part of the authors.

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