

RESEARCH ARTICLE

High expression of DARS2 indicates poor prognosis in lung adenocarcinoma

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Abstract

Background: DARS2 was overexpressed in multiple tumor types, but the biological role of DARS2 in lung adenocarcinoma (LUAD) have not been elucidated.

Methods: Firstly, the DARS2 expression in LUAD was explored using The Cancer Genome Atlas (TCGA). Then, qRT-PCR and Western blot were performed to confirm DARS2 expression in LUAD. Next, Cox regression and Kaplan–Meier methods were utilized to evaluate whether DARS2 expression can affect the overall survival. The relationships between DARS2 expression and clinicopathological characteristics were investigated by TCGA database. Moreover, we utilized Gene Set Enrichment Analysis (GSEA) to detect DARS2-related signaling pathways in LUAD. Finally, the special function of DARS2 in cell proliferation, invasion and apoptosis was assessed in vitro.

Results: The higher expression of DARS2 was found in LUAD compared to paracarcinoma tissues and significantly related to tumor stage, T stage, and M stage. The survival analysis indicated that DARS2 overexpression was related to poor prognosis in LUAD. Multivariate analysis suggested that DARS2 expression was a prognostic indicator. GSEA revealed that DARS2 was primarily involved in cell cycle-related pathways. In addition, upregulation of DARS2 facilitated LUAD cell proliferation, migration, invasion and inhibited apoptosis, DARS2 knockdown showed an opposite result.

Conclusion: DARS2 modulates the proliferation, invasion and apoptosis of LUAD cells, and sever as a promising therapeutic target for LUAD.

KEYWORDS

apoptosis, DARS2, lung adenocarcinoma, prognosis, tumorigenesis

1 | INTRODUCTION

Lung cancer ranks as the leading cause of morbidity and mortality of all malignancies in the world, and the trend is rising year by year.¹ Non-small-cell lung cancer (NSCLC), which accounts for nearly 80% of lung cancers, is the leading type of lung cancer, and lung

adenocarcinoma (LUAD) is the dominant subtype.² While major breakthroughs have been achieved in therapies of LUAD in the past decades, the clinical outcomes of advanced-stage lung cancer have turned out to be worse than expected.³ Cause of delayed diagnosis, high recurrence rate, and drug resistance, the 5-year overall survival (OS) rate remains nearly 15%.^{4,5} Hence, exploring the potential

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molecular mechanisms and find out more effective therapeutic strategies for lung cancer are urgent need.

Aminoacyl-tRNA synthetases (ARSs) are critical enzymes which synthesize proteins by catalyzing amino acids with homologous transfer RNA (tRNAs).⁶ ARSs has long been thought to be a relatively evolutionarily conserved housekeeping protein with no additional function.⁷ However, emerging studies have shown that ARSs have evolved a wild type of functions apart from the protein synthesis, and plays an important role in angiogenesis, cell homeostasis, and tumorigenesis.⁸⁻¹¹ Aspartyl-tRNA Synthetase 2 (DARS2), encoded by the class-II aminoacyl-tRNA synthetase family gene, is a mitochondrial enzyme which specifically aminoacylates aspartyl-tRNA.¹² DARS2 is located in chromosome 1q25.1, which mainly exists in brain stem, spinal cord, liver.¹³⁻¹⁵ DARS2 is a vital member of the ARSs family and shares many similar physiological and pathological functions with other members. N'Gbo et al.¹⁵ reported that DARS2 gene mutations could reduce the catalytic activity of aspartyl-tRNA synthase, which affected the synthesis of extra proteins in mitochondria. Qin et al.¹⁶ showed that the overexpression of DARS2 significantly accelerated hepatocellular carcinoma tumorigenesis and was associated with a poor prognosis. Cheng et al. reported that a prognostic model for bladder cancer based on DARS2 and other genes, which explored and confirmed DARS2 as an independent prognostic factor.^{17,18} Zhang et al.¹⁹ suggested that DARS2 was considered as a novel biomarker for acute leukemia using the bioinformatics analysis. Nevertheless, the role of DARS2 in LUAD has rarely been investigated previously.

In this study, we investigated the DARS2 expression pattern in LUAD and corresponding para-carcinoma tissues and explored the association between DARS2 expression and overall survival. In addition, the assay of DARS2 knockdown and overexpression were conducted to explore the effect on tumor proliferation, invasion and apoptosis. All the findings confirmed that DARS2 could be regarded as a novel biomarker for LUAD therapy.

2 | MATERIALS AND METHODS

2.1 | Tissue samples

Between May 2019 and May 2020, 45 pairs of LUAD and para-carcinoma tissues were collected from patients with undergone pneumonectomy at the department of Thoracic Surgery, Fujian Provincial Hospital. The specimens had complete clinical data and did not receive chemotherapy before pneumonectomy. This clinical research was supported by the Ethics Committee of Fujian Provincial Hospital. Informed consent was gained from each patient.

2.2 | RNA extraction and qRT-PCR

Total RNA was isolated from tissues and cells using TRIzol method (Invitrogen). cDNA was synthesized by the Prime Script RT Reagent

kit (TAKARA). mRNA expression level was detected using a Light Cycler 480 System (Applied Roche) and TB Green Premix Ex Taq™ (TAKARA). The primers used were as follows: DARS2 5'-GGAGAGTT GCGTTCGTCTCA-3' (forward), 5'-GATTCACAGGG GCTTCACA-3'(reverse). The gene expression was normalized using β -actin.

2.3 | Cell culture

NSCLC cell lines (A549 and H1299) were obtained from John Minna (UT Southwestern), and cultured in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% FBS at 37°C in 5% CO₂.

2.4 | Cell proliferation assay

The CCK-8 Kit (Beyotime Technology, China) was used to evaluate cell viability. The transfected cells (5.0×10^3 /ml) were placed into 96-well plates and cultured in incubator (37°C, 5% CO₂). After 0, 24, 48, and 72h cultivation, 100 μ l CCK-8 reagent was added in each well, and absorbance was detected at 450 nm.

2.5 | Transwell assay

For the migration assay, the 2×10^6 /ml cells were diluted by serum-free medium containing 1% FBS, followed by 100 μ l cell suspension was seeded to upper chamber, and 500 μ l medium containing 10% FBS was added to bottom chamber. After 24h incubation, cells were immobilized with paraformaldehyde and stained with crystal violet. The only difference in invasion assay was that we pre-spread Matrigel (BD Biosciences) evenly in the upper membranes. Finally, five fields were randomly selected and counted using a microscopy.

2.6 | Apoptosis analysis in flow cytometry

The transfected cells were seed in 6-well plates for 24h. Afterward, cells were gathered by trypsinization without EDTA and washed twice using PBS. The resuspended cells were stained with Annexin V- PI Apoptosis Detection Kit (Beyotime) according standard procedures. The apoptosis rate was tested by flow cytometry.

2.7 | Plasmid construction and transfection

The DARS2 plasmid and empty vector (NC) were designed by RiBoBio. The lentivirus of DARS2/NC was purchased from Genepharma (Shanghai, China). A549 and H1229 cells were added to 6-well plates on the day before transfection, and then lentivirus was transfected into cells at appropriate MOI. Polybrene (Maobai) was utilized to improve the transfection efficiency. After incubation for 12h, the medium was replaced with fresh medium. At 72h

transfection, the cells were identified and harvested for further experiments.

2.8 | siRNA interference and transfection

The siRNA was obtained from zolgene (Zolgene). The following target sequence was used to silence DARS2 expression: siRNA-1438, 5'-AGAGUUUAAUUGAGGGUUUTT-3' (sense) and 5'-AAACCCUCAAUUAACACUCUTT-3' (antisense); siRNA-1699, 5'-GGAAAGACAUUGAAUCCAUTT-3' (sense) and 5'-AUGGAUUCAAUGUCUUUCCTT-3' (antisense); siRNA-1373, 5'-AGACAGACAGCCUGAGUUUTT-3' (sense) and 5'-AAACUCAGGCUGUCUGUCUTT-3' (antisense), and negative control siRNA sequences were 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACAGUUCGGAGAATT-3' (antisense). The transfection was conducted by Lipofectamine 2000 (Invitrogen) for 48 h.

2.9 | Western blot analysis

All tissue proteins were extracted by RIPA lysis buffer (Beyotime). Protein concentration was detected by BCA method (Beyotime) before SDS-PAGE. Twenty micrograms of protein was separated by SDS-PAGE, transferred to PVDF membranes, and blotted with primary antibodies against following proteins: DARS2 (Abcam), β -actin (Abcam). The members were incubated by primary antibodies at 4°C overnight and HRP-conjugated secondary antibodies (Proteintech) were incubated at room temperature for 1 h.

2.10 | Bioinformatics analysis

The RNA-seq data and corresponding clinical characters from 538 LUAD patients were searched and downloaded from TCGA database (<https://cancergenome.nih.gov>). In this study, survival analysis based on TCGA and GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) was applied to investigate the relationship between DARS2 expression and overall survival. Then, we performed Cox regression analysis to explore correlation between DARS2 expression and clinical characters. Additionally, GSEA was utilized to predict the underlying mechanisms of DARS2.

2.11 | Statistical analysis

Statistical evaluations were processed with GraphPad Prism 6.04. Measurement data were presented as the mean \pm SD. Student's test and Pearson's correlation analysis were performed for statistical comparisons. The OS curves were estimated by the Kaplan–Meier method. Significance was set at $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).

3 | RESULTS

3.1 | DARS2 overexpression is significantly associated with poor prognosis of LUAD

The findings from TCGA database discovered that DARS2 mRNA expression level was significantly overexpressed in LUAD (Figure 1A). Similarly, we observed that DARS2 was markedly increased in LUAD tissues compared to matched para-carcinoma tissues (Figure 1B). Next, qRT-PCR analysis on 45 pair LUAD tissues revealed that the overexpression of DARS2 in LUAD compared with the para-carcinoma tissues (Figure 1C). Moreover, Western blot was carried out to examine DARS2 expression on 8 pair randomly selected LUAD tissues. As shown in Figure 1D, the results displayed that DARS2 protein expression was observably upregulated in LUAD compared with para-carcinoma tissues. Moreover, the GSEA analysis further confirmed that DARS2 was enriched in the pathway of "NON-SMALL LUNG CANCER" (Figure 1E).

Next, survival analysis was conducted based on TCGA and GEO database. The data suggested that an upregulated expression level of DARS2 was related to poor survival in LUAD patients (Figure 1F–H). Additionally, Cox regression analysis were performed to explore whether DARS2 was a prognostic indicator for LUAD. Univariate Cox regression analysis suggested that the DARS2 expression was related to OS (HR = 1.040, $p = 0.0085$; Table 1). Multivariate Cox regression analysis identified that the DARS2 expression was an independent prognostic factor for OS (HR = 1.056, $p = 0.0455$), and clinical stage (HR = 1.931, $p = 0.0037$; Table 1). All data suggested that the overexpression of DARS2 was related to worse survival in LUAD patients.

3.2 | Relationship between DARS2 expression and clinicopathological parameters in LUAD

Then, the connection between DARS2 expression and clinicopathological characters in LUAD from TCGA database was explored. The results indicated that DARS2 expression was remarkably elevated in male compared to female ($p = 1.615e-06$; Figure 2B). In addition, high DARS2 expression was significantly associated with clinical stages (Figure 2C, $p = 0.003$), T stages (Figure 2D, $p = 0.021$) and M stages (Figure 2F, $p = 7.616e-04$). However, there was no significant difference between DARS2 expression and age and N stage (Figure 2A,E).

3.3 | DARS2 knockdown inhibits LUAD cells proliferation, migration, invasion, and promotes cell apoptosis

To explore the effects of DARS2 on cells growth and invasiveness, we knockdown the DARS2 expression using three targeted siRNAs (si-DARS2 438, si-DARS2 1373, and si-DARS2 1699) in A549 and

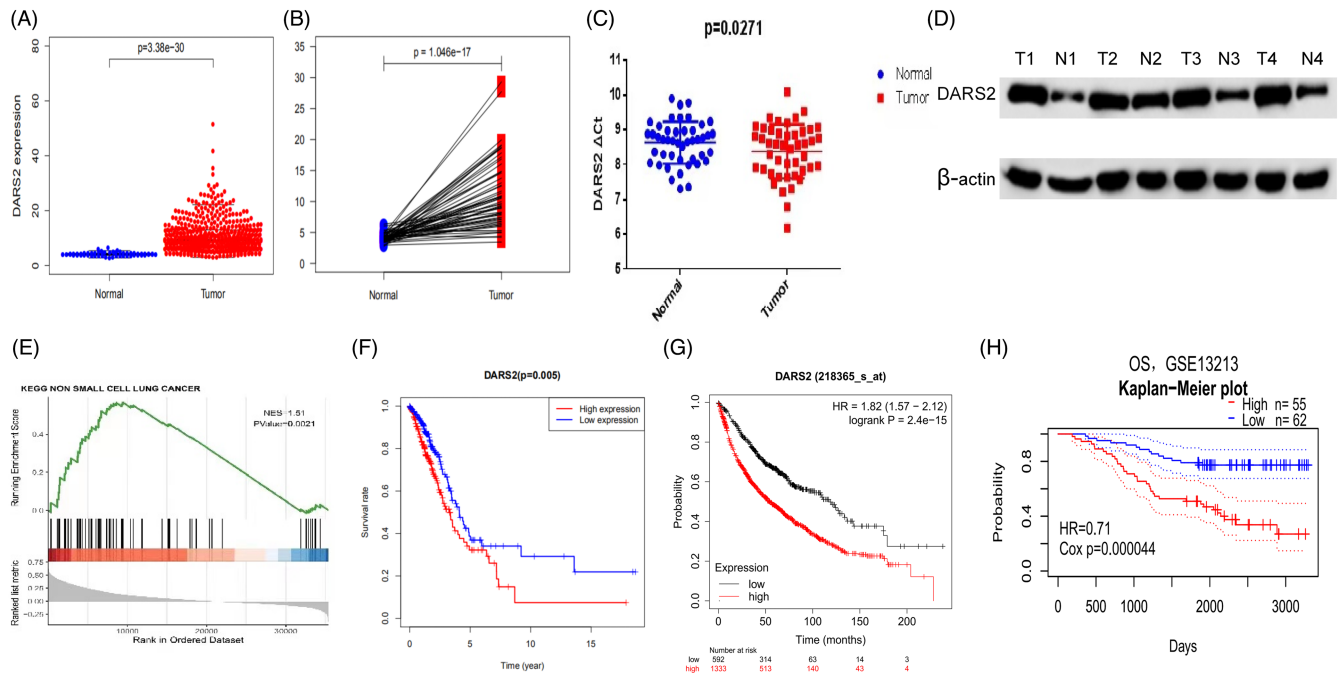


FIGURE 1 DARS2 overexpression was related to worse prognosis in LUAD. (A, B) DARS2 mRNA expression from TCGA Lung Cancer dataset. (C) qRT-PCR assay was performed to analyze DARS2 expression from paired LUAD and para-carcinoma tissues. (D) Western blot was conducted to detect the DARS2 expression in five paired LUAD and para-carcinoma tissues. (E) GSEA revealed that DARS2 was enriched in the pathway of "NON-SMALL CELL LUNG CANCER." (F-H) Survival analysis based on TCGA and GEO database showed that DARS2 overexpression was associated with poor prognosis.

Variable	Univariate analysis		Multivariate analysis	
	HR (95%CI)	p value	HR (95%CI)	p value
Age				
≥60 vs. <60	1	0.9290		
Gender				
Male vs. Female	1	0.9958		
Clinical stage				
Stage III-IV vs. Stage I-II	1.644	<0.001	1.931	0.0037
T grade				
T3-T4 vs. T1-T2	1.623	<0.001	1.170	0.2707
M grade				
M1 vs. M0	1.681	0.0891		
N grade				
N2-N3 vs. N0-N1	1.793	<0.001	1.024	0.9005
SFXN1 expression				
High expression vs. Low expression	1.040	0.0085	1.056	0.0455

Abbreviations: CI, confidence interval; HR, hazard ratio.

TABLE 1 Univariate and multivariate Cox regression analysis of different prognosis factors in patients with LUAD from TCGA database

H1299 cells. The results of qRT-PCR and Western blot revealed that siRNA-DARS2 markedly reduce the expression of DARS2 (Figure 3A). Due to the most obviously interference efficiency, the sequence of si-DARS2 1373 was selected to silence DARS2 in the loss of function experiments. The CCK-8 assays suggested that silencing DARS2 significantly reduced cell viability (Figure 3B).

Transwell assays also revealed that a significant restriction in si-DARS2 groups compared to NC groups (Figure 3D). Furthermore, flow cytometry results showed that DARS2-knockdown cells had higher apoptotic rates than NC cells (Figure 3C). All findings proved that silencing DARS2 reduced the LUAD cells tumorigenicity in vitro.

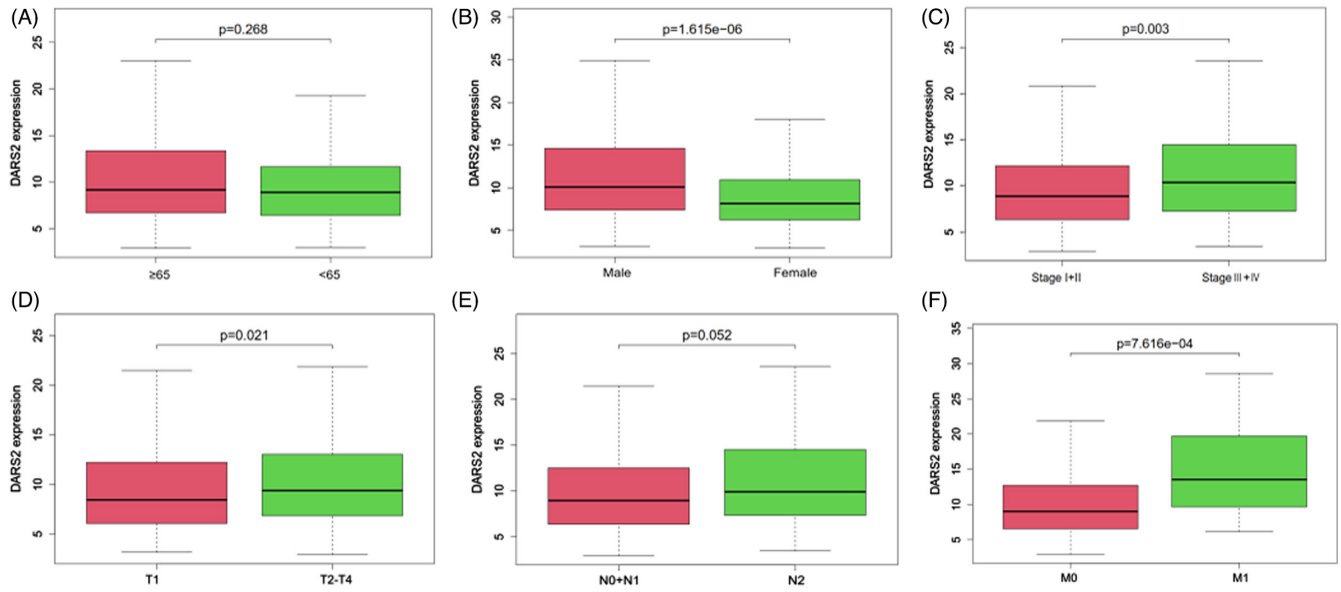


FIGURE 2 Relationship between DARS2 expression and clinicopathological characters in LUAD. (A) DARS2 expression in different age. (B) DARS2 expression in different sex. (C) DARS2 expression in tumors with different clinical stages. (D-F) DARS2 expression in tumors with different T, N, and M stages.

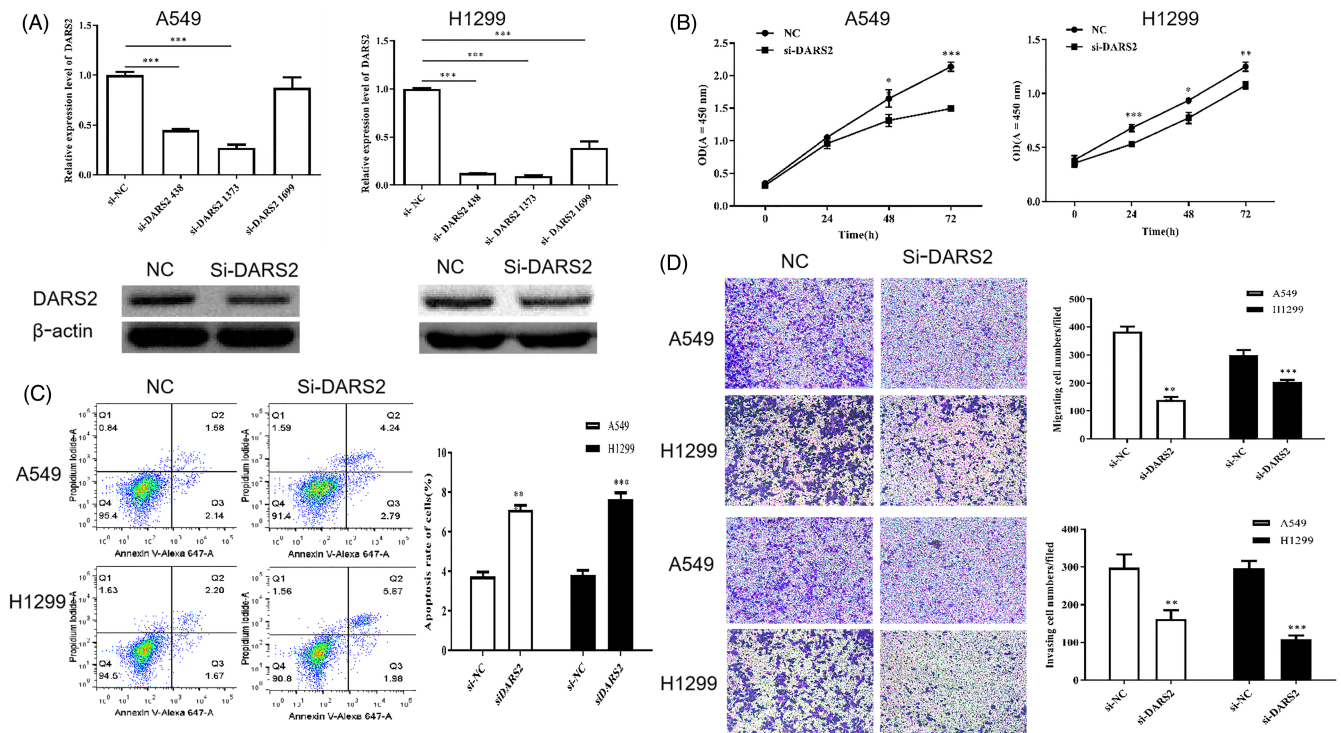


FIGURE 3 Knockdown of DARS2 decreased cell proliferation, migration, invasion, and facilitated apoptosis in LUAD cells. (A) qRT-PCR and Western blot analysis were conducted to examine DARS2 expression in A549 and H1299 cells transfected with siRNAs. (B) CCK-8 assay was utilized to detect the A549 and H1299 cell viability. (C) Flow cytometry was conducted to measure apoptotic rate of A549 and H1299 cells. (D) The effect of DARS2 silence on the migration and invasion of A549 and H1299 cells was investigated by transwell assays.

3.4 | DARS2 overexpression facilitates LUAD cells proliferation, migration, invasion, and reduced apoptosis

To further identify whether DARS2 expression could affect biological functions of NSCLC cells, the DARS2 overexpressed and controlled lentiviral vector were transfected to the cells. We performed CCK-8 assay to examine LUAD cells viability. The cells transfected by OE-DARS2 exhibited a greater viability compared to the NC groups (Figure 4A). As revealed in Figure 4C,D, DARS2 overexpression markedly promoted cell migration and invasion. Moreover, we analyzed apoptotic rate using flow cytometry in DARS2 overexpressing cells. These data indicated that the percentage of apoptosis obviously reduced in OE-DARS2 groups compared with the NC groups (Figure 4B). Taken together, the data suggested that overexpression of DARS2 promoted tumorigenicity and suppressed apoptosis of LUAD cells *in vitro*.

3.5 | GSEA identified DARS2-related signaling pathways

To clarify the potential mechanisms activated by DARS2 in LUAD, we performed the GSEA analysis on DARS2 differential expression datasets. KEGG pathways showed that asthma, cell adhesion molecules, cell cycle, hematopoietic cell lineage, cytokine receptor interaction, intestinal immune network for IgA production, lysine degradation, one carbon pool by folate, mismatch repair, and

RNA degradation were most associated with DARS2 expression (Figure 5A). GO terms revealed that asymmetric cell division, interleukin 13 production, macrophage migration, negative regulation of DNA replication, negative regulation of DNA repair, regulation of macrophage migration, chromosome centromeric region, chromosome centromeric region, cytokine receptor activity, immune receptor was significantly related to DARS2 expression (Figure 5B). Collectively, the results demonstrated that DARS2 might play a vital role in progression and gene regulation of LUAD.

4 | DISCUSSION

Due to the poor outcomes and easy metastasis, it is essential to identify new prognostic markers for LUAD.¹⁹ A number of studies have confirmed that AARS are involved in RNA splicing, transcription and translation regulation, cell apoptosis, and tumorigenesis in higher eukaryotes.^{20–22} The DARS2 gene encodes mitochondrial aspartyl-tRNA synthetase, which is essential to the progression of HCC.¹⁶ However, the role of DARS2 in LUAD has remained largely unknown.

In this work, DARS2 expression pattern and its prognostic value in LUAD were analyzed. First, we analyzed DARS2 expression in LUAD and para-carcinoma tissues from TCGA database and discovered that DARS2 was notably upregulated in LUAD. Next, qRT-PCR and Western blot were conducted to confirm the DARS2 overexpression in LUAD. Furthermore, DARS2 upregulation was accompanied by elevation of tumor grade and clinical stage. Kaplan–Meier

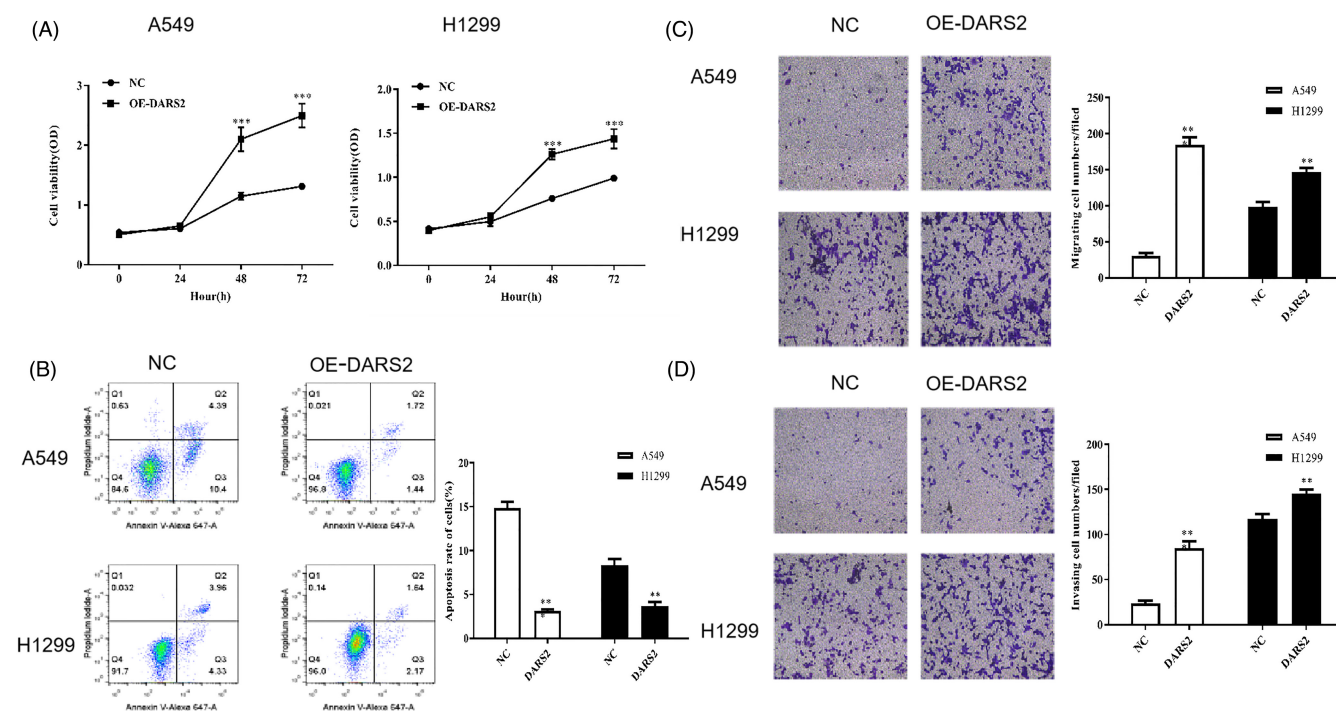


FIGURE 4 DARS2 overexpression enhanced proliferation, migration, invasion, and inhibited apoptosis in LUAD cells. (A) The effect of DARS2 overexpression on cell proliferation of A549 and H1299 cells were estimated by CCK-8 assay. (B) Cell apoptosis rate was analyzed by the flow cytometry. (C–D) Cell invasion and migration were examined by transwell assays in DARS2 overexpressing A549 and H1299 cells.

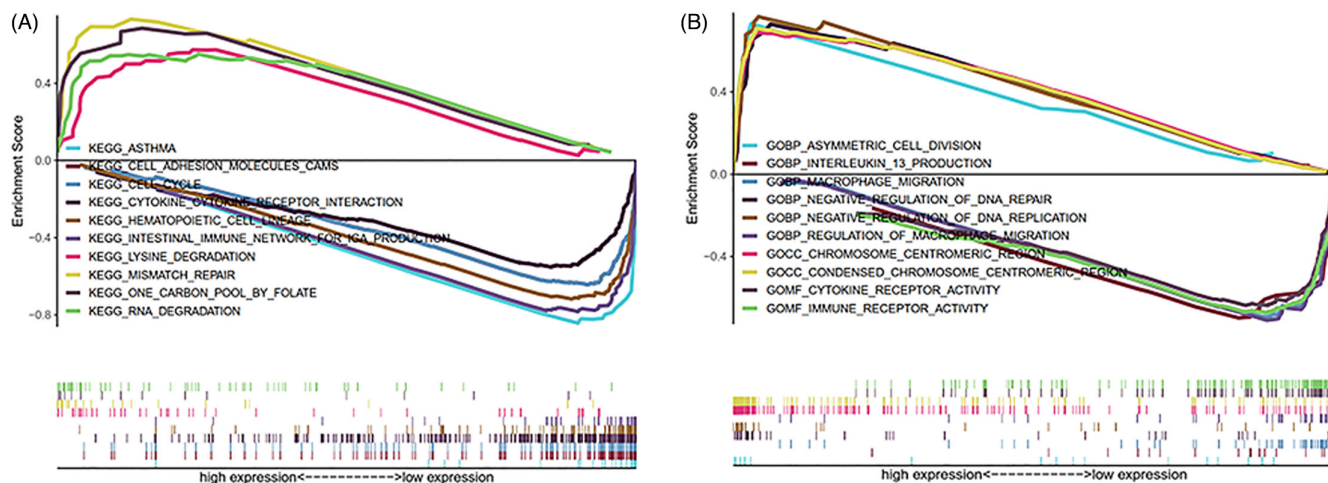


FIGURE 5 Gene set enrichment analysis (GSEA) of DARS2 in LUAD. (A, B) Gene set enrichment plots were constructed to display the enriched pathways associated with DARS2 according to GO and KEGG analyses

curves from different database demonstrated that high DARS2 expression level was related to worse survival in LUAD. Univariate analysis suggested that DARS2 and multiple clinical factors, including clinical stage and TNM stage, might indicate a poor outcome. Additionally, multivariate analysis further identified that DARS2 was a prognostic indicator. The findings revealed that DARS2 was a diagnostic and prognostic indicator in LUAD.

In terms of function, silencing DARS2 could restrict the LUAD cell proliferation, migration, and invasion, whereas DARS2 overexpression showed an opposite result. Qin et al demonstrated that DARS2 facilitated cell cycle progression and suppressed hepatocellular carcinoma cell apoptosis via the miR-30e-5p/MAPK/NFAT5 pathway.¹⁶ To predict the role of DARS2 in LUAD, we performed GSEA to examine signaling pathways associated with DARS2 in LUAD. The results revealed that DARS2 primarily involved in cell cycle-related pathways. It was reported that AspRS were crucial for tRNA synthesis and various cellular processes, including cytokine and immune responses.²³ Our findings were basically consistent with the above research. To sum up, DARS2 is an oncogene that promotes cell proliferation, invasion, and suppress apoptosis. Nonetheless, more research is needed to focus on the mechanism by which DARS2 participates in LUAD development. Nevertheless, there are some limitations to this study, more clinical cases should be enrolled in this work. Second, there are lack of functional tests in vivo. Third, the mechanism of DARS2 in LUAD development is still not well understood.

In conclusion, our findings confirmed that DARS2 might serve as a potential oncogene in LUAD. DARS2 was highly expressed in LUAD tissues. Elevated DARS2 expression corresponded to shorter overall survival. DARS2 overexpression facilitated the LUAD cell cycle progression and suppressed apoptosis. This highlighted DARS2 as a diagnostic marker and therapeutic target of LUAD.

AUTHOR CONTRIBUTIONS

Liangyuan Chen and Wenbing Wu designed the research. Yingfeng Jiang, Jianbin You, Chuncai Wu and Yanli Kang carried out the

experimental work. Yingfeng Jiang, Yanli Kang, Falin Chen, and Liangyuan Chen analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

INFORMED CONSENT

Written informed consent was obtained from each patient.

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