Hypoxia-mediated YTHDF2 expression and activation of the mTOR/AKT axis in lung squamous cell carcinoma

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Research Article

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Abstract

Background

N6-methyladenosine (m6A) is a dynamic and reversible internal RNA structure of eukaryotic mRNA. YTH domain family 2 (YTHDF2), an m6A-specific reader YTH domain family, plays fundamental roles in several types of cancer. However, the function of YTHDF2 in lung squamous cell carcinoma (LUSC) remains elusive.

Methods

Functionally, NCI-H226 and SK-MES-1 cells were exposed to hypoxia to detect the protein levels of hypoxia-inducible factor-1α (HIF-1α), endogenous YTHDF2, and phospho-AKT (Ser473) analyzed by western blotting and then the association of these proteins with LUSC was analyzed with a bioinformatics database. Next, we established stable YTHDF2 upregulation models in NCI-H226 and SK-MES-1 cells to explore the function of YTHDF2 in LUSC cells by performing in vitro and in vivo assays. Finally, we affirmed that YTHDF2 overexpression was involved in activating the mTOR/AKT signaling and inducing the EMT process in LUSC using western blotting. Clinically, immunohistochemical staining revealed the relationship between YTHDF2 expression levels and the clinicopathological characteristics of lung squamous cell carcinoma patients.

Results

The results showed that hypoxia-mediated YTHDF2, a tumor promoter, promoted cell proliferation and invasion by activating the mTOR/AKT axis and inducing the EMT process in LUSC. Moreover, YTHDF2 was closely associated with pN (pN− 37.0%, pN+ 73.9%; P = 0.002) and pTNM stage (PI 50.0%, PII 43.3%, pIIIa 80.6%; P = 0.007), ultimately resulting in poor survival for LUSC patients.

Conclusion

In brief, the results highlight the critical role of YTHDF2 in both hypoxia exposure and the pathogenesis of LUSC.

Introduction

Worldwide, malignant tumors of the lung are the primary cause of cancer incidence and death, ranking as the highest tumor-related mortality with more than 1.8 million deaths in 2018 and accounting for almost 1 in 5 cancer deaths [1]. Lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD) are the most common histological subtypes of non-small cell lung cancer (NSCLC), which accounts for almost 80%-85% of all human lung cancers. With the development of targeted drugs for specific gene...
mutations, this treatment has recently greatly improved the clinical prognosis of advanced LUAD patients recently. In contrast, LUSC patients have a poor clinical prognosis and lack targeted agents compared to LUAD patients [2–4]. Therefore, there is an urgent need to search for new oncogenic drivers to inhibit the development and progression of LUSC patients.

The cellular response to hypoxia, followed by activation of hypoxia-inducible factor 1 (HIF-1), has been reported to be emerging as an important mechanism promoting tumor aggressiveness, metastasis, and poor prognosis [5]. N6-methyadenosine(m6A), the most prevalent modification of mRNA, is not only induced by hypoxia and promotes cancer progression, angiogenesis, and metastasis in several cancers [6, 7], but is also widely involved in many biological processes, such as splicing and stability of mRNA, RNA nucleation, the interaction between RNA and protein, and protein translation [8–10]. To date, hypoxia, as an attractive therapeutic target, has not been successfully exploited in the lung [11]. Consequently, it is critical to further investigate the molecular mechanism of hypoxia exposure and the prognosis of lung squamous cell carcinoma.

In addition, m6A RNA modification acts as a dynamic and reversible internal RNA modification process promoted by a ‘writer’ complex (METTL3, METTL14, WTAP, and other undiscovered subunits), inhibited by ‘erasers’ (FTO and ALKBH5), and functionally executed by ‘readers’ (the YTH domain-containing family (YTHDF1-3, YTHDC1-2) and HNRNP family proteins) [12–14]. Studies documenting the “reader” proteins indicate that they mainly mediate the fates of m6A modified mRNAs, such as mRNA processing, translation and degradation, microRNA (miRNA) processing, and nuclear export [8, 15–17]. Recognition by the IGF2BP family can enhance the target mRNA stability [18]. m6A-containing transcript translation was promoted by a combination of YTHDF1 and YTHDF3 [19–21]. Furthermore, YTHDF2 could regulate m6A-modified mRNA degradation [10]. In summary, the biological functions of m6A modification in mRNA have been reported to contribute to regulating the progression of cancer development [22–24].

More than 90% of cancer-related mortality is associated with cancer cell metastasis [25]. Epithelial-mesenchymal transition (EMT) plays an important function in cell migration, invasion, and cancer progression, endowing cells with stem cell properties and contributing to immunosuppression [26]. Furthermore, Snail superfamily members, the prominent inducers in EMT, are very strongly implicated in tumor grade, recurrence, metastasis, and poor prognosis in various tumors types [27, 28]. Recently, it has been reported that RNA epigenetic factors mediate EMT progression and the development of cancer [29]. For example, adverse prognosis in liver patients was associated with the coregulation of METTL3 and YTHDF1. Moreover, studies highlight the key role of m6A in EMT progression, cancer metastasis, and YTHDF1-mediated Snail translation [30]. The roles of m6A in the EMT process and Snail expression need to be further investigated in other cancers. Certainly, our findings indicated that upregulation of YTHDF2 induces the EMT process, promotes cancer metastasis, and predicts a worse prognosis in LUSC patients.

It has been reported that YTHDF2 expression is promoted in multiple tumors including lung cancer patients [31–33]. In this study, we further explored the expression level and biological role of YTHDF2 in lung squamous cell cancer. Our data showed that YTHDF2 was mediated by hypoxia exposure and
orchestrated proliferation and invasion in lung squamous cell cancer. Mechanistically, hypoxia-stimulated HIF-dependent upregulation of YTHDF2 resulted in the promotion of the EMT process and activation of the mTOR/AKT signaling pathway. Thus, we hope that this study can further explore the molecular mechanism of hypoxia exposure and provide potential therapeutic targets for LUSC.

**Methods**

**Cell culture**

In a humidified incubator (37°C, 5% CO2), NCI-H226 and SK-MES-1 cells from the American Tissue Culture Collection (ATCC), were cultured in DMEM medium (KeyGEN BioTECH, Nanjing, China), and 10% fetal bovine serum (Gibco, United Kingdom) and 1% penicillin-streptomycin solution (KeyGEN BioTECH, Nanjing, China) were added. Using shRNA lentiviral particles (GENECHEM, Shanghai, China) containing YTHDF2, two cell lines were infected according to the infection instructions to achieve the stable upregulation of exogenous YTHDF2. The upregulation of exogenous protein expression was detected by western blotting.

**Western blotting and antibodies**

Total proteins were extracted from cultured cells by RIPA buffer. The results showed that the BCA detection method (Beyotime, Nantong, Jiangsu, China) quantitatively detected all proteins. The protein samples separated by PAGE (polyacrylamide gel electrophoresis) were transferred to the PVDF membranes (EMD Millipore, Billerica, Massachusetts, USA). The PVDF membranes were blocked in 5% skim milk for 1 hour, then incubated with specific antibodies overnight at 4°C, and finally incubated with the secondary antibodies for 2 hours at room temperature. The membranes were visualized using enhanced chemiluminescence solution (Pierce Biotechnology, Inc. USA). The experimental results were analyzed by ImageJ analysis software. The following antibodies were recorded: anti-GAPDH, anti-Flag-tag, anti-HIF1α, anti-YTHDF2 (Proteintech, Wuhan, Hubei, China), anti-AKT, anti-Phospho-AKT (Ser473), anti-ERK1/2, anti-Phospho-ERK1/2 (Thr202/Tyr204, Cell Signaling Technologies, Danvers, MA, USA), anti-Phospho-mTOR (Ser2448), anti-mTOR, anti-E-cadherin, anti-N-cadherin, anti-Vimentin, anti-Snail1, anti-METTL3, anti-METTL14.

**Cell proliferation assay**

Cell Counting Kit 8 (CCK-8) was used to detect cell proliferation. A total of 2000 cells per well were plated in 96-well plates (Costar; USA). At the indicated time points, 10 μL of CCK-8 reagent (Dojindo) was added to the cells, and the cells were cultured in a humidified incubator (37°C, 5% CO2) for another 1 h. Then the optical density at A490 nm was measured by an enzyme-linked immunosorbent assay (ELISA) reader.

**Migration and invasion assays**
The wound-healing assay measured cell migration activity. NCI-H226 and SK-MES-1 cells (4 x 10^6) were inoculated into six-well plates. After 80% of the cells were fused, 1.3-mm-wide scratched wounds were washed with PBS, and then photographed at 0 and 24 hours. The transfected cells were plated in the upper chamber of Transwell Matrigel chambers (Collaborative Biomedical Products, USA) at 1.2 x 10^4 per chamber. We added serum-free medium and 10% serum-containing medium to the upper and lower layers of the chamber respectively. After 24 hours, the invasive cells were fixed, stained, photographed, and quantified. The stained cells were counted in five random fields at ×100 magnification, and the average number was taken.

**Animal experiments**

The animal experiment was approved by the Animal Research Committee of Jinan Central Hospital Affiliated with Shandong University.

Thirty-five-day-old male nude mice (athymic BALB/c-nu) were obtained from Shandong University (Jinan, China). The mice were randomly divided into two groups which were inoculated with stable YTHDF2-expressing LUSC cells and the vector LUSC cells. A total of 5 x 10^6 of the cells were suspended in 0.1 ml of PBS and then injected subcutaneously into the flanks of mice. Tumor size was measured twice a week. The volume formula was: width^2 x length x π/6. After 5 weeks, well-trained individuals performed physical methods of euthanasia: cervical dislocation on nude mice in a familiar and safe environment, then the xenograft tumor load was isolated, photographed, weighed, and fixed in formalin to perform immunohistochemical staining of YTHDF2.

**Tissue samples and immunohistology**

From October 2008 to May 2013, 73 LUSC patients were included in this study at the Department of Jinan Central Hospital Affiliated with Shandong University. We included patients with LUSC diagnosed after complete surgical resection and postsurgical pathology. TNM staging was performed according to the 8th edition of the IASLC Lung Cancer Staging Project [34]. We have obtained informed consent to conduct experiments on human subjects. This study was approved by the Ethics Committee of Jinan Central Hospital Affiliated with Shandong University.

All the LUSC specimens and the adjacent normal lung tissue came from 73 patients. Tissue samples were fixed with a 10% neutral formalin and treated routinely. After dewaxing, inactivation of endogenous peroxidase, and antigen repair, 5% BSA blocking solution was added for 30 minutes at 37 °C. Then, the sections were incubated with YTHDF2 antibody (1:200, Catalog #A02621-1, BOSTER, Wuhan, China) overnight at 4°C and secondary antibody (Ready-to-use SABC-POD (rabbit IgG) Kit, Catalog # SA1022, BOSTER, Wuhan, China) at 37°C for 30 minutes. The specimens were developed with diaminobenzidine (DAB) and stained with Mayor’s hematoxylin at 37°C for 1 minute. Finally, the specimens were analyzed by ImageScope software (Leica) and histochemistry scores were obtained. The median histochemistry score was used to divide patients into two groups.
Statistical analysis

The experimental data were analyzed by Student’s t-test, the chi-squared test, or Fisher’s exact probability test. GraphPad Prism 8.3.0 and the IBM SPSS Statistics 25 were used to perform statistical analyses. Kaplan-Meier survival curves were used in univariate analysis and a Cox predictive risk model was used in multivariate analysis. All the data came from three independent experiments, in triplicate. Moreover, P values less than 0.05 were considered statistically significant.

Results

**Hypoxia specifically induces YTHDF2 expression in LUSC cells.**

Hypoxia induces hypoxia-inducible factor-1α (HIF-1α), which is mediated by a proline hydroxylase and has emerged as a crucial factor. Moreover, hypoxia is associated with poor prognosis and resistance to radiation and chemotherapy [35].

First, we investigated whether hypoxic exposure affected YTHDF2 expression in LUSC cells. NCI-H226 and SK-MES-1 cells were exposed to 1% oxygen for 24 hours. The expression of HIF-1α was used to validate the hypoxic response. We found that endogenous YTHDF2 expression was increased approximately 2- or 3-fold at 24 h after hypoxia exposure, respectively. Interestingly, the level of AKT that was phosphorylated at serine 473 was increased approximately 3- or 11-fold at 24 h following hypoxia (Fig. 1A and 1B). Meanwhile, we employed bioinformatics-based screening to explore the association of HIF-1α, YTHDF2, and AKT1 in human lungs. LUSC data mining of the GEPIA [http://gepia.cancer-pku.cn/] that HIF-1α expression was correlated with AKT1 and YTHDF2 (Fig. 1C and D). As expected, the results presented a statistically positive correlation between the expression of YTHDF2 and AKT1 (Fig. 1E). In summary, the protein level of YTHDF2 in LUSC cells was specifically increased under hypoxia, and YTHDF2 mediated activation of the mTOR/AKT signaling pathway.

**YTHDF2 upregulation promotes LUSC cell proliferation and invasion.**

We established stable YTHDF2 upregulation models in NCI-H226 and SK-MES-1 cells to explore the biological function of YTHDF2 in LUSC. Successful overexpression of YTHDF2 was confirmed at the protein level (Fig. 2A and 2B). The CCK-8 assay was assessed cellular viability. As shown in Fig. 2C and Fig. 2D, cellular viability dramatically increased in the YTHDF2 group compared with that of the cells carrying the vector only. Moreover, to explore the effects of YTHDF2 upregulation on cell motility, we examined the invasion potential of NCI-H226 and SK-MES-1 cells by using a Transwell assay. As expected, YTHDF2 overexpression dramatically promoted LUSC cell invasion abilities (Fig. 2E and Fig. 2F). Next, the wound-healing assay showed that overexpression of YTHDF2 dramatically strengthened the migratory capabilities of LUSC cell lines (Fig. 2G and Fig. 2H). Moreover, a subcutaneous implantation experiment in nude mice was performed to investigate the oncogenic function of YTHDF2 in LUSC. Compared to those bearing the vector only, we showed that stable upregulation of YTHDF2 markedly promoted tumor growth in nude mice, as demonstrated by the significant increase in tumor size.
and weight (Fig. 3A, 3B, 3C, 3D, 3E, and 3F). In the immunohistochemical results, the YTHDF2 expression levels of the xenograft tumors using the indicated stable cells were higher than those carrying the vector only (Fig. 3G and Fig. 3H). Hence, our data suggested that YTHDF2 plays a critical role in promoting LUSC proliferation and invasion.

**YTHDF2 facilitates the mTOR/AKT signaling cascades and induces EMT in LUSC cells.**

We investigated whether YTHDF2 overexpression activates those pivotal signaling pathways in LUSC cells, such as the ERK/MAPK and mTOR/AKT signaling pathways which are known to function in tumor proliferation and survival. The results showed that the phosphorylation of both AKT and mTOR was markedly increased in the YTHDF2 group compared with that of the cells carrying the vector only, but not of ERK (Fig. 4A). Furthermore, YTHDF2 expression was positively correlated with AKT and mTOR in LUSC according to the GEPIA [http://gepia.cancer-pku.cn](http://gepia.cancer-pku.cn) (Fig. 1E and 4C). Taken together, YTHDF2 upregulation promotes cell proliferation by activating the mTOR/AKT pathway rather than the ERK/MAPK pathway in LUSC.

Meanwhile, phosphorylation of mTOR and AKT by YTHDF2 promotes its activity, stabilizing Snail1 to repress the expression of E-cadherin. As a tumor suppressor, the E-cadherin change elicited by YTHDF2 upregulation was in line with the activation of the mTOR/AKT pathway. Specifically, the downregulation of E-cadherin was accompanied by the upregulation of N-cadherin in the EMT process, which altered cell adhesion (Fig. 4B). Therefore, our study indicated that the upregulation of YTHDF2 induced the EMT process, and promoted cancer metastasis in LUSC cells.

**YTHDF2, as a tumor promoter, may lead to a poor prognosis for LUSC patients.**

Recently, an increasing number of studies have examined the correlation analysis between writer proteins and reader proteins [36, 37]. Our data suggested that the overexpression of YTHDF2 directly affected the expression level of METTL14 at the protein level (Fig. 5A). Using the GEPIA [http://gepia.cancer-pku.cn](http://gepia.cancer-pku.cn), we also found a marked correlation between the expression of YTHDF2 and METTL14, but not METTL3 in LUSC (Fig. 5B and 5C). Therefore, we speculated that YTHDF2 cooperating with METTL14 may be involved in LUSC.

Using the GEPIA [http://gepia.cancer-pku.cn](http://gepia.cancer-pku.cn) to detect the overall survival (OS) and the disease-free survival (DFS) of genes (YTHDF2, Snail, METTL14), the results showed that LUSC patients at the mRNA level with increased expression of Snail had poor DFS and OS, but all not of YTHDF2, while the upregulation of METTL14 only lessened DFS (Fig. 5D). In the immunohistochemical staining results, YTHDF2 protein expression levels in lung squamous cell carcinoma tissues were markedly higher than those in the corresponding normal lung tissues (Table 1 and Fig. S1). We further explored the correlation between YTHDF2 expression and clinicopathological characteristics. The 5-year survival rate of the 73 LUSC patients accounted for 39.7%. Table 2 shows that the YTHDF2 upregulation was markedly correlated with pN (pN− 37.0%, pN+ 73.9%; P=0.002) and pTNM stage (pI 50.0%, pII 43.3%, pIIIa 80.6%; P=0.007). According to the log-rank test with univariate analysis, the 5-year survival rate of LUSC patients
was closely related to pN (P=0.001), pTNM stage (P=0.01), and high expression of YTHDF2 (P=0.002, Table 3). Ultimately, Cox regression with multiple analyses showed that pN and YTHDF2 expression acted as independent factors affecting the 5-year survival rate (Table 4). Consistently, these data showed that YTHDF2, which induced the high expression of METTL14 and Snail, may lead to a worse prognosis for LUSC patients.

**Discussion**

In this study, we suggested that YTHDF2 upregulation was significantly induced by hypoxia in LUSC cells. Overexpression of YTHDF2 positively activated the mTOR/AKT pathway and regulated the progression of EMT which may act as a tumor promoter to induce LUSC cell proliferation and invasion.

Generally, it has been reported that m6A modification is associated with tumorigenesis. A better explanation of the molecular mechanisms of a complete m6A modification process requires the cooperation of m6A writer genes, erasers genes, and readers genes, rather than a single isolated gene. For example, m6A modification mediated by the cooperation of METTL14, ALKBH5, and YTHDF3 was reported to influence the cell cycle, induce the progression of EMT, and contribute to angiogenesis of cancer cells in breast cancer [37]. YTHDF1-mediated the translation of Snail was verified, as a portion of EMT was altered by deletion of METTL3 in liver patients [30]. In bladder cancer, the mutual interaction between METTL3 and YTHDF2 induced the degradation of SETD7 and KLF4 mRNA in the proliferation and metastasis process [32]. Moreover, the methyltransferase METTL3 was discovered to regulate the degradation of SOCS2 mRNA, enhancing the progression of liver cancer in a YTHDF2-mediated m6A-dependent manner [36]. Here, we also demonstrated that YTHDF2 was positively related to METTL14 and cooperated with METTL14 in LUSC. However, how this mutual collaboration between METTL14 and YTHDF2 activates the oncogenic signaling pathway in LUSC is largely unknown. We will address the unknown mechanism in our next study.

In our study, the YTHDF2 protein expression level was markedly higher in human LUSC tissues than in normal tissues. Cell proliferation was significantly enhanced in YTHDF2-overexpression cells compared with control cells. Moreover, YTHDF2 upregulation promoted tumor growth and increased tumor volume in vivo compared with control cells. The mechanism underlying YTHDF2-mediated LUSC tumorigenesis was also investigated. The results indicated that compared with control cells, AKT and mTOR phosphorylation was significantly increased following YTHDF2 overexpression, which is crucial for tumor progression. Based on the results, it was hypothesized that YTHDF2 may promote LUSC cell proliferation by activating the AKT/mTOR signaling pathway which is known to play a pivotal role in multiple types of cancer, such as breast cancer and ovarian carcinoma [38, 39]. To the best of our knowledge, the present study is the first study to demonstrate the role of YTHDF2 in LUSC cell proliferation. However, our present study failed to analyze AKT and mTOR expression levels by immunohistochemistry.

Our study was not without limits: for instance, there are reports that YTHDF2 regulates m6A-modified mRNA degradation, which is seemingly contrary to our study. One possible explanation is that YTHDF2
decays the m6A-modified mRNA of tumor suppressor genes, thus promoting cell growth. Another possible explanation is that YTHDF2-mediated mRNA decay might not be the only mechanism underlying m6A function in cancer progression. In addition, one report suggests that PI3K-AKT signaling is involved in promoting the EMT process via the mTOR or MAPK cascade [40], and another report indicates that the m6A modification is associated with the EMT progression and cancer metastasis that is induced by YTHDF1-mediated Snail translation in liver patients [38]. Therefore, the existence of these two signaling axes remains to be verified in LUSC, and whether m6A mediates E-cadherin expression via other factors/pathways also deserves further exploration. In future studies, we will contribute to addressing these unresolved limitations.

In summary, the present study indicated that YTHDF2 was involved in mediating LUSC cell proliferation and invasion. These results may improve the current understanding of the mechanism underlying the biological role of YTHDF2 during tumor development and might provide a potential therapeutic target for LUSC.

Abbreviations

m6A: N6-methyladenosine; YTHDF2: YTH domain family 2; LUSC: lung squamous cell carcinoma; HIF-1α: Hypoxia-inducible factor-1α; LUAD: lung adenocarcinoma; NSCLC: non-small cell lung cancer; miRNA: microRNA; EMT: the epithelial-mesenchymal transition; ATCC: the American Tissue Culture Collection; PAGE: polyacrylamide gel electrophoresis; CCK-8: Cell Counting Kit 8; ELISA: enzyme-linked immunosorbent assay; DAB: diaminobenzidine; OS: the overall survival; DFS: the disease-free survival.

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Declarations

Ethics approval and consent to participate
All procedures performed in studies involving human participants were under the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments, or comparable ethical standards. Informed consent was obtained from all individual participants included in the study. The Ethics Committee of Jinan Central Hospital Affiliated to Shandong University has approved conducting this study.

Consent for publication
The participant has consented to the submission of the research to the journal.

Consent to participate
Informed consent was obtained from all individual participants included in the study.

Availability of data and material
All data are included in this article.

**Conflicts of interest**

All authors declare no conflict of interest.

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**Authors' contributions**

Nan Zhang and Zhi-Gang Sun designed this work. Peng Xu wrote the manuscript and prepared the figures and tables. Kang Hu drafted and revised the manuscript. All the authors contributed to manuscript revision, read and approved the submitted version.

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None

**Tables**

**Table 1.** YTHDF2 expression in LUSC compared with para-carcinoma tissue

| Group            | n  | YTHDF2 expression | P       |
|------------------|----|-------------------|---------|
|                  |    | Low (n%)          | High (n%)|
| LUSC             | 73 | 29 (39.7)         | 44 (60.3) | 0.000*** |
| para-carcinoma   | 73 | 58 (79.5)         | 15 (20.5) |

P-value: chi-squared test

**Table 2.** Relationship between YTHDF2 and clinicopathological factors of LUSC patients
| Clinicopathological parameters | N       | YTHDF2 Low (n=29) | YTHDF2 High (n=44) | P       |
|--------------------------------|---------|-------------------|--------------------|---------|
| Gender                        |         |                   |                    | 0.298   |
| Male                          | 66      | 28                | 38                 |         |
| Female                        | 7       | 1                 | 6                  |         |
| Age                           |         |                   |                    | 0.337   |
| <60                           | 23      | 11                | 12                 |         |
| ≥60                           | 50      | 18                | 32                 |         |
| Differentiation               |         |                   |                    | 0.550   |
| Good                          | 3       | 0                 | 3                  |         |
| Moderate                      | 53      | 22                | 31                 |         |
| Poor                          | 17      | 7                 | 10                 |         |
| pT classification             |         |                   |                    | 0.901   |
| pT1                           | 12      | 4                 | 8                  |         |
| pT2                           | 32      | 13                | 19                 |         |
| pT3                           | 29      | 12                | 17                 |         |
| pN                             |         |                   |                    | 0.002   |
| -                              | 27      | 17                | 10                 |         |
| + pTNM stage                  | 46      | 12                | 34                 | 0.007   |
| pI                             | 12      | 6                 | 6                  |         |
| pII                            | 30      | 17                | 13                 |         |
| pIII                           | 31      | 6                 | 25                 |         |

P-value: chi-squared test, Fisher’s exact probability test. pT classification, tumor size; pN, lymph node metastasis and pTNM stage, tumor stage.

Table 3. Results of univariate analysis concerning 5-year survival of the LUSC patients
| Clinical features      | Patients (N=73) | 5-year survival (%) | P     |
|------------------------|----------------|---------------------|-------|
|                        | patients      | Rate (%)            |       |
| Gender                 | 0.711         |                     |       |
| Male                   | 66            | 26                  | 39.4  |
| Female                 | 7             | 3                   | 42.9  |
| Age                    | 0.451         |                     |       |
| ≥60                    | 23            | 10                  | 43.5  |
| <60                    | 50            | 19                  | 38.0  |
| Differentiation        | 0.928         |                     |       |
| Good                   | 3             | 2                   | 66.7  |
| Moderate               | 53            | 21                  | 39.6  |
| Poor                   | 17            | 6                   | 35.3  |
| pT classification      | 0.465         |                     |       |
| pT1                    | 12            | 6                   | 50.0  |
| pT2                    | 32            | 13                  | 40.6  |
| pT3                    | 29            | 10                  | 34.5  |
| pN                     | 0.001         |                     |       |
| -                      | 27            | 19                  | 70.4  |
| +                      | 46            | 10                  | 21.7  |
| pTNM stage             | 0.001         |                     |       |
| pI                     | 12            | 9                   | 75.0  |
| pII                    | 30            | 15                  | 50.0  |
| pI\a                   | 31            | 5                   | 16.1  |
| YTHDF2 expression      | 0.002         |                     |       |
| Low                    | 29            | 16                  | 55.2  |
High 44 13 29.5

P – Log-rank test; pT classification, tumor size; pN, lymph node metastasis and pTNM stage, tumor stage.

Table 4. Results of Cox multivariate regression 5-year survival rate of the LUSC patients

| Clinical features       | B    | SE   | Wald | P     | HR   | 95.0%CI for HR |
|-------------------------|------|------|------|-------|------|----------------|
| Differentiation         | 0.616| 0.343| 3.219| 0.073 | 1.851| 0.945~3.626    |
| pT classification       | 0.274| 0.250| 1.195| 0.274 | 1.315| 0.805~2.147    |
| pN                      | 1.669| 0.650| 6.588| 0.010 | 5.309| 1.484~18.997   |
| pTNM stage              | 0.254| 0.403| 0.397| 0.528 | 1.289| 0.585~2.840    |
| YTHDF2 expression       | 0.781| 0.355| 4.856| 0.028 | 2.184| 1.090~4.377    |

B, regression coefficient; SE, standard error; Wald, Wald value; HR, hazard ratio; CI, confidence interval; pT classification, tumor size; pN, lymph node metastasis and pTNM stage, tumor stage.

Figures

![Figure 1](image-url)

**Figure 1**
Hypoxia specifically induces YTHDF2 expression in LUSC cells. (A) and (B) NCI-H226 and SK-MES-1 cells were exposed with or without 24h hypoxia (1% O2, 5%CO2, 94% N2). The protein levels of HIF-1α, YTHDF2, and P-AKT (be phosphorylated at serine 473) were analyzed by western blot. Data are represented by the mean ± SD of three independent experiments. *P<0.05 vs. the vector group. Using the Pearson correlation statistics, we examine the pairwise gene correlation analysis between HIF-1α and AKT1 (C), HIF-1α and YTHDF2 (D), YTHDF2 and AKT1 (E) by TCGA and GTEx expression data of GEPIA.

Figure 2
YTHDF2 upregulation promotes LUSC cell proliferation and invasion. (A) and (B) Representative immunoblot showing the protein level of YTHDF2 stable upregulate expression in two LUSC cell lines studied. (C) and (D) Cell viability was assessed by the CCK8 assay in NCI-H226 and SK-MES-1 cells. (E) and (F) Cell invasion potential of NCI-H226 and SK-MES-1 cells was assessed by the transwell cell invasion assay. (G) and (H) The cell migration ability of NCI-H226 and SK-MES-1 cells was evaluated by the wound-healing assay. Data are represented by the mean ± SD of three independent experiments. *P<0.05 vs. the vector group.

Figure 3

The LUSC tumorigenicity of YTHDF2 in nude mice. (A, B, C, D) After a month, we dissected tumors from nude mice which had been injected with the indicated stable cell. (E, F) we measured the tumor's size and weight of nude mice injected by the indicated stable cells twice a week. Data are represented by the mean ± SD of three independent experiments. *P<0.05 vs. the vector group. (G) and (H) Immunohistochemical showing of YTHDF2 from the tumors of nude mice injected by the indicated stable cells.

Figure 4

YTHDF2 facilitates mTOR/AKT signaling cascades and induces EMT in LUSC cells. (A) The western blot detected the expression of mTOR/AKT-related proteins in NCI-H226 and SK-MES-1 cells. (B) The western blot detected the expression of EMT-related proteins in NCI-H226 and SK-MES-1 cells. Data are represented by the mean ± SD of three independent experiments. *P<0.05 vs. the vector group. (C) Using the Pearson correlation statistics, we examine the pairwise gene correlation analysis between YTHDF2 and mTOR by TCGA and GTEx expression data of GEPIA.
Figure 5

YTHDF2, as a tumor promoter, may lead to a poor prognosis for LUSC patients. (A) Representative immunoblot showing YTHDF2 stably upregulated expression to promote METTL14 upregulation, not of METTL3 in NCI-H226. Data are represented by the mean ± SD of three independent experiments. *P<0.05 vs. the vector group. (B) and (C) Using the Pearson correlation statistics, we examine the pairwise gene correlation analysis between YTHDF2 and METTL14, YTHDF2 and METTL3 by TCGA and GTEx.
expression data of GEPIA. (D) By a log-rank test for the overall survival (OS) and disease-free survival (DFS) analysis in LUSC, we respectively investigate gene YTHDF2, METTL14, and Snail by the ‘Survival’ tab of GEPIA. (E) In the immunohistochemical staining results, the overall survival of LUSC patients with pN, pTNM, and YTHDF2 expression was analyzed by A Kaplan-Meier analysis.

Supplementary Files

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