GRP75-Upregulated HMGA1 Expression Promotes Stage I Lung Adenocarcinoma Progression by Activating the JNK/c-JUN Signaling Pathway

Guo-Bing Qiao
Third Military Medical University Daping Hospital and Research Institute of Surgery

Ren-Tao Wang
PLAGH: Chinese PLA General Hospital

Shu-Nan Wang
Third Military Medical University Daping Hospital and Research Institute of Surgery

Shaolin Tao
Third Military Medical University Daping Hospital and Research Institute of Surgery

Qun-You Tan
Third Military Medical University Daping Hospital and Research Institute of Surgery

Hua Jin (✉️ 13527538470@163.com)
Third Military Medical University Daping Hospital and Research Institute of Surgery

https://orcid.org/0000-0002-1988-8009

Research

Keywords: HMGA1, GRP75, stage I LUAD progression, JNK/c-JUN signaling

DOI: https://doi.org/10.21203/rs.3.rs-122104/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background:** Recurrence is a major challenge in early-stage lung adenocarcinoma (LUAD) treatment. However, the recurrence mechanism is still unclear, and no biomarkers can predict recurrence in early-stage LUAD. Here, we investigated the role and mechanism of high-mobility group AT-hook 1 (HMGA1) and glucose-regulated protein 75-kDa (GRP75) in stage I LUAD and evaluated their potential as biomarkers for predicting the recurrence and prognosis of stage I LUAD.

**Results:** High expression of HMGA1 and GRP75 was associated with recurrence and a poor prognosis in stage I LUAD patients. In particular, HMGA1 had potential as an independent prognostic factor. Overexpression of GRP75 or HMGA1 promoted LUAD cell growth and metastasis, while silencing GRP75 or HMGA1 inhibited LUAD cell growth and metastasis. *In vitro* and clinical data showed that the expression level of GRP75 positively regulated HMGA1 in LUAD and that GRP75 played an HMGA1-dependent role. In addition, GRP75 prolonged the half-life of HMGA1 by inhibiting HMGA1 ubiquitination via direct binding to HMGA1. Finally, we demonstrated that the GRP75/HMGA1 axis played a role by activating JNK/c-JUN signaling in LUAD.

**Conclusions:** The activation of GRP75/HMGA1/JNK/c-JUN signaling is an important mechanism that promotes the progression of stage I LUAD, and a high level of HMGA1 is a novel biomarker for predicting recurrence and prognosis in patients with stage I LUAD.

**Background**

Lung cancer is the most malignant human cancer with the highest incidence and mortality worldwide[1]. Lung adenocarcinoma (LUAD) is a type of non-small cell lung cancer (NSCLC) that accounts for 40% of all lung malignancies[2] and surgery is the major treatment method for patients with early-stage LUAD[3, 4]. However, 30%~40% of stage I LUAD patients ultimately die because of locoregional or metastatic recurrence after surgery[5]. Unfortunately, no biomarker can accurately predict postoperative recurrence in patients with early-stage LUAD, and the postoperative progression mechanism of early-stage LUAD is currently unclear.

High-mobility group AT-hook 1 (HMGA1) is an architectural transcription factor[6]. Studies show that HMGA1 expression is abnormally upregulated in several types of cancer, including lung cancer, and HMGA1 acts as an oncogene[7–9]. Notably, various studies indicate that HMGA1 is involved in the regulation of epithelial-mesenchymal transition (EMT) and cancer stem cells (CSCs)[10–12]. EMT is a key step in cancer metastasis because it is a process by which epithelial tumor cells lose their cell polarity and cell-cell adhesion and gain migratory and invasive properties[13]. Additionally, a study showed that recurrence is accompanied by EMT in breast cancer[14]. Additionally, accumulating evidence shows that CSCs are another key factor in cancer metastasis and recurrence[15–17]. CSCs are a small subset of cells within tumors that behave like stem cells. Studies show that CSCs have high tumorigenicity, are able to undergo self-renewal, and can regenerate all cell types in a tumor, resulting in tumor relapse[18].
Importantly, clinical data also show that the expression levels of the CSC marker protein ALDH1 and CD133 are closely correlated with recurrence and a poor prognosis in patients with early-stage NSCLC[19]. Together, these findings suggest that HMGA1 may be involved in the progression of early-stage lung cancer. However, the upregulation mechanism and the functional role of HMGA1 in the progression of early stage LUAD have not been reported.

In the present study, we demonstrated that HMGA1 stimulated LUAD cell viability and metastasis and a high expression level of HMGA1 was an independent prognostic indicator closely correlated with recurrence and a poor prognosis in patients with stage I LUAD. In addition, we found that the upregulated expression of HMGA1 was caused by increased expression of GRP75 in LUAD. Additionally, we identified that GRP75 was associated with recurrence and a poor prognosis in stage I LUAD and played an oncogenic role that was HMGA1 dependent. Finally, we demonstrated that the GRP75/HMGA1 axis promoted LUAD progression by activating JNK/c-JUN signaling.

Materials And Methods

Materials

Fetal bovine serum (FBS) and cell culture medium were purchased from HyClone (South Logan, UT, USA). MG132 and kanamycin were purchased from Sigma (St. Louis, MO, USA). JNKIN8 was obtained from MedChem Express (Shanghai, China). Plasmids for overexpressing HMGA1 or GRP75 and HMGA1-specific short hairpin RNA (shRNA) were designed and synthesized by GeneChem (Shanghai, China). Small interfering RNA (siRNA) oligonucleotides targeting HMGA1 or GRP75 were obtained from RiboBio (Guangzhou, China). A cell counting kit-8 (CCK8) kit was purchased from Bioss (Beijing, China). A migration assay kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). Cell invasion assay chambers (24 wells) were purchased from Corning (NY, USA). Cycloheximide (CHX) and primary antibodies against HMGA1, GRP75, Ki67, Ubiquitin, JNK, P-JNK (Thr183/Tyr185), c-JUN, P-c-JUN (Ser63) and P-c-JUN (Ser73) and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). A Pierce coimmunoprecipitation kit was obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Cell lines, cell transfection and RNA interference

The lung adenocarcinoma cell lines A549 and PC9 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS at 37 °C in a humidified atmosphere with 5% CO₂.

Cells were transfected with the indicated plasmids or siRNAs using Lipofectamine 3000 (Invitrogen, CA) according to the manufacturer’s protocol. The siRNA sequences against HMGA1 were as follows: 5’-GAAGTGCCAACACCTAAAGA-3’ and 5’-AGCGAAGTGCCAACACCTA-3’. The siRNA sequences against GRP75 were as follows: 5’-TGCCATGTCTTAGACAAGA-3’ and 5’-GGATTGTCACTGATCTAAT-3’. The control siRNA sequence was 5’-UUCUCGGACGUGUCAGUTT-3’. The HMGA1-specific short hairpin RNA sequence was 5’-GATCCCTGCTACACCTTGTGCCACTTGGTGCTGTTAGCACTTTTTTGAT-3’, and the
control sequence was 5′-TTCTCGAACGTGCACGT-3′. After transfection for 48 or 72 hours, the cells were subjected to further analyses.

**Transwell and cell viability assays**

The migratory and invasive abilities of LUAD cells were assessed with a transwell assay. The indicated cells were transfected with the indicated oligonucleotides or plasmids for 48 h. Then, $3 \times 10^4$ cells in serum-free growth medium were seeded in the upper chambers. The lower chambers were filled with the same medium supplemented with 10% FBS. After 24 h, the cells that had invaded the lower side of the chamber were fixed with 4% paraformaldehyde (PFA), stained with 0.1% crystal violet and dried. The number of invading cells was counted under a light microscope.

For cell viability assays, the indicated cells were plated in 96-well plates at $4 \times 10^3$ cells per well. At the indicated time points, cell viability was estimated by using CCK8 reagent according to the manufacturer's instructions. Absorbance was measured at a wavelength of 450 nm by using a microplate reader.

**Colony formation assays**

After 48 h of transfection, cells were trypsinized and resuspended in 0.5 ml 0.35% agar in growth medium at a density of 1000 cells/well (24-well plate). Then, the agar-cell mixture was plated on top of a solid layer of 0.8% agar in the growth medium. The cells were cultured until visible clones appeared (approximately 3 weeks). The cell clones were washed twice with PBS, fixed with methanol for 20 min, stained with 0.1% crystal violet for 1 h, washed with PBS, and counted.

**Western blot analysis**

Western blot analysis was carried out according to a standard protocol. Cell lysates were generated from cultured cells with RIPA lysis buffer containing protease inhibitors (Roche). The protein concentration was determined by using a Bradford kit (Pierce, Thermo Fisher Scientific). Equal amounts of total protein were loaded, separated by SDS-PAGE and then transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). After blocking in 5% nonfat milk, the membranes were incubated overnight at 4 °C with appropriate primary antibodies and then with secondary antibodies conjugated to horseradish peroxidase for 2 hours at room temperature. After washing, the bands of interest were analyzed by using an ECL kit. Images were acquired using the Bio-Spectrum Gel Imaging System (Bio-Rad, USA). Then, quantification of the Western blot results was performed using Image Lab software, and the bands were normalized with GAPDH as the internal control.

**Immunoprecipitation and mass spectrometry**

Immunoprecipitation was performed using a Thermo Fisher Scientific Pierce coimmunoprecipitation kit following the manufacturer's protocol. Briefly, the indicated antibodies were first immobilized for 2 h using AminoLink Plus coupling resin. The resin was then washed and incubated with lysate overnight at 4 °C. After the incubation, the resin was washed, and proteins were eluted using elution buffer. A negative control that was provided with the co-IP kit to assess nonspecific binding received the same treatment as
the samples with IgG. The immunoprecipitated samples were resolved by SDS-PAGE, followed by Western blotting with appropriate antibodies.

For liquid chromatography-mass spectrometry (LC-MS) analysis, samples, which included those with HMGA1 overexpression and a control, were separated using electrophoresis, and specific bands were identified using a mass spectrometry system; the molecules corresponding to the bands were identified in a human proteomic library to obtain proteins directly bound to HMGA1.

**Degradation and deubiquitylation assay**

To detect HMGA1 degradation mediated by GRP75 in A549 cells, cells with or without GRP75 overexpression were treated with CHX (CST, 100 µg/ml) for the indicated durations before analysis. Then, cell lysates were analyzed using Western blotting with an anti-HMGA1 antibody. To detect HMGA1 deubiquitination in A549 cells, cells were transfected with the indicated plasmids and treated with 20 µM proteasome inhibitor MG132 (Sigma) for 8 h before harvest. Then, the cells were lysed in IP lysis/wash buffer and incubated with the anti-HMGA1 antibody, which was used for IP. The immunoprecipitated samples were resolved by SDS-PAGE, followed by Western blotting with an anti-ubiquitin antibody.

**Bioinformatic analysis**

Publicly available databases were used to evaluate gene expression and predict potential mechanisms. A clinical dataset of stage I LUAD patients was obtained from the TCGA database and analyzed using TIMER[20]. mRNA-sequencing analysis was performed by Huada Genomics Institute (BGI, Guangzhou, China), and KEGG pathway analysis was conducted with the Dr. Tom program (https://biosys.bgi.com).

**Animal experiments**

Animal studies were conducted at Daping Hospital, according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal experiments were approved by the Animal Research Committee of Army Medical University. Six-week-old female nude mice were utilized. To investigate the effects of oncogenic HMGA1 on LUAD cell tumorigenicity, the indicated cells were transfected with HMGA1 or HMGA1-specific shRNA-expressing or control plasmids. After 72 h of transfection, 2 × 10^6 cells in 0.1 mL PBS were injected subcutaneously (five mice per group). Tumor length (L) and width (W) were measured weekly, and tumor volume (V) was calculated using the formula $V = L \times W^2 / 2$. Four weeks after cell injection, these mice were sacrificed, and the tumors were harvested and weighed. To investigate the presence of Ki67 proteins in mouse tumor tissues, an immunohistochemistry assay was performed as described previously[21]. For metastatic ability experiments, four groups of five mice each were given tail vein injections of 2 × 10^6 the indicated cells, respectively. Four weeks after injection, all mice were sacrificed, the tumor nodules formed on the lung surface were counted. The lungs were embedded with paraffin and sliced for HE staining.

**Statistical analysis**
Results

Upregulated expression of HMGA1 is associated with recurrence and a poor prognosis in stage I LUAD patients

First, we used a TCGA dataset to investigate whether the expression level of HMGA1 is involved in the recurrence of stage I LUAD. As in previous reports, the TCGA analysis results showed that HMGA1 expression was abnormally upregulated in many types of cancer tissue, including LUAD tissue, compared to the corresponding normal tissue (Fig. 1a and b). Notably, the expression level of HMGA1 in stage I LUAD tissues was significantly higher than that in normal lung tissues (Fig. 1c), and the high expression level of HMGA1 in primary tumors was closely correlated with recurrence in patients with stage I LUAD (Fig. 1d). In addition, the TCGA dataset analysis results showed that the stage I LUAD patient group with a high level of HMGA1 had a shorter median recurrence-free survival (RFS) time (Fig. 1e) and lower overall survival (OS) rate than the low HMGA1 expression group (Fig. 1f). Collectively, these results indicate that the increased expression level of HMGA1 is closely correlated with recurrence and a poor prognosis in stage I LUAD patients.

Upregulation of HMGA1 expression stimulates LUAD progression and is a therapeutic target in LUAD

Next, we investigated whether the upregulated expression of HMGA1 directly stimulates LUAD progression. We first generated a construct expressing green fluorescent protein (GFP)-tagged HMGA1 and transfected it into LUAD cells (Fig. 2a), we then performed cell viability, soft agar colony formation and transwell assays. Our results showed that overexpression of HMGA1 significantly stimulated cell viability (Fig. 2b), soft agar colony formation (Fig. 2c), migration and invasion (Fig. 2d) in both A549 cells and PC9 cells. These in vitro results were further confirmed in vivo using xenograft models. As shown in Fig. 2e and 2f, tumor growth and tumor weight were remarkably higher in the HMGA1 overexpression group than in the vector control group. Additionally, an increased expression level of the cell proliferation marker protein Ki67 was detected in HMGA1-overexpressing LUAD tissues (Fig. 2g). Consistent with the subcutaneous animal experiments, the mice in the HMGA1 group had more and larger lung metastatic nodules were observed compared to the vector control (Fig. 2h and 2i). These findings suggest that upregulated expression of HMGA1 directly stimulate LUAD progression.

These findings prompted us to investigate whether HMGA1 silencing can suppress LUAD progression. As expected, silencing of HMGA1 (Fig. 3a) significantly suppressed LUAD cell viability (Fig. 3b), soft agar
colony formation (Fig. 3c), migration and invasion in vitro (Fig. 3d). Consistently, animal experiments also showed that silencing HMGA1 expression (Additional file 2: Fig S1) dramatically suppressed tumor growth (Fig. 3e and f), tumor cell proliferation (Fig. 3g) and metastasis (Fig. 3h and 3i). These findings indicate that inhibition of HMGA1 may be a useful strategy for treating LUAD.

**Upregulated protein expression of HMGA1 was caused by increased expression of GRP75 in LUAD**

To elucidate the regulatory and functional mechanism of HMGA1 overexpression in LUAD, we identified proteins that bind to HMGA1 in LUAD cells using IP/LC-MS assays. Our results identified 25 proteins that might specifically interact with HMGA1 in LUAD cells (Figs. 4a and b, Additional file 1: Table S1). Among them, GRP75 was selected for further study, as GRP75 was previously reported to promote metastasis and be closely associated with early recurrence in liver cancer[22]. The interaction between GRP75 and HMGA1 in LUAD was further confirmed using coimmunoprecipitation (co-IP) assays (Fig. 4c, Additional file 3: Fig S2). Notably, TCGA data analyses showed that GRP75 protein levels were positively correlated with HMGA1 protein levels in stage I LUAD tissues (Fig. 4d). Since the role of GRP75 in early-stage LUAD is not clear, we then investigated the effect of GRP75 on early-stage LUAD using a TCGA dataset analysis and in vitro experiments. As shown in Fig. 4e-g, the TCGA dataset analysis showed that GRP75 was highly expressed in stage I LUAD tissues compared to normal tissues (Fig. 4e) and that high expression levels of GRP75 were closely associated with recurrence (Fig. 4f) and shortened RFS in patients with stage I LUAD (Fig. 4g). In addition, the in vitro experiments showed that overexpression of GRP75 significantly stimulated LUAD cell viability (Fig. 4h), migration and invasion (Fig. 4i), while silencing GRP75 significantly suppressed LUAD cell viability (Fig. 4j), migration and invasion (Fig. 4k). These findings suggest that GRP75 acts as an oncoprotein in early-stage LUAD and may be a partner protein of HMGA1.

Next, we investigated how HMGA1 and GRP75 affect each other's expression and functions in LUAD cells. Our Western blot data showed that HMGA1 overexpression or silencing did not affect GRP75 protein levels, while GRP75 overexpression or silencing upregulated or downregulated HMGA1 protein levels in LUAD cell lines, respectively (Fig. 5a). In addition, in vitro experiments showed that silencing of HMGA1 significantly inhibited the GRP75 overexpression-induced stimulation of LUAD cell viability (Fig. 5b), invasion and migration (Fig. 5c). Interestingly, multivariate analysis identified HMGA1 but not GRP75 as an independent prognostic predictor in stage I LUAD (Table 1). Importantly, RFS analysis showed that there was no correlation between a high expression level of GRP75 and recurrence in stage I LUAD patients with low expression of HMGA1 (Fig. 5d). Taken together, these findings suggest that HMGA1 is a downstream protein of GRP75 and that GRP75 promotes HMGA1-dependent early-stage LUAD progression.
### Table 1

Multivariate analyses for recurrence-free survival survival by Cox regression model

| Parameters            | Multivariate analysis |
|-----------------------|-----------------------|
|                       | HR        | 95% CI     | P          |
| Age                   | 1.658     | 0.769–3.575 | 0.197      |
| Sex                   | 0.994     | 0.515–1.918 | 0.986      |
| Smoking history       | 1.100     | 0.804–1.504 | 0.552      |
| Stage                 | 1.688     | 0.848–3.360 | 0.136      |
| HMGA1 expression      | 2.486     | 1.249–4.948 | 0.009      |
| GRP75 expression      | 0.973     | 0.492–1.921 | 0.936      |

Further, we investigated the mechanism by which GRP75 upregulates HMGA1 expression in LUAD. Unlike our protein data, our mRNA data showed that the mRNA expression level of HMGA1 was not affected by GRP75 overexpression or silencing (Fig. 5e), suggesting that GRP75 regulates HMGA1 expression through posttranscriptional regulation. Because GRP75 binds directly to HMGA1, we speculated that GRP75 may participate in the regulation of HMGA1 protein degradation. To confirm this hypothesis, we investigated the effect of GRP75 on the half-life of the HMGA1 protein in LUAD cells treated with the protein synthesis inhibitor cycloheximide (CHX). Compared with the vector control, GRP75 overexpression robustly prolonged the half-life of the endogenous HMGA1 protein in A549 cells (Fig. 5f). In addition, the ubiquitination of HMGA1 in A549 cells was dramatically inhibited by GRP75 overexpression (Fig. 5g). These findings suggest that GRP75 upregulates the HMGA1 protein level by protecting the HMGA1 protein from ubiquitination-mediated degradation.

**GRP75/HMGA1 axis stimulates LUAD progression by activating the JNK/c-JUN signaling pathway**

To further investigate the molecular mechanisms by which the GRP75/HMGA1 axis stimulates the progression of early-stage LUAD, we performed mRNA sequencing using HMGA1- or GRP75-overexpressing LUAD cells and their corresponding control cells (Fig. 6a). Then, KEGG signaling pathway enrichment analysis was performed using a set of genes that similarly upregulated or downregulated in cells with overexpression of HMGA1 and GRP75 compared to their corresponding control. As shown in Fig. 6b, we found that the JNK signaling pathway was correlated with overexpressed HMGA1 and GRP75 in LUAD. In addition, our Western blot analysis showed that overexpression of HMGA1 stimulated the phosphorylation of JNK (Thr183/Tyr185) and its downstream protein c-JUN (Ser63 and Ser73) in LUAD cells, whereas HMGA1 silencing inhibited the phosphorylation of both JNK (Thr183/Tyr185) and c-JUN (Ser63 and Ser73) (Fig. 6c). Consistent with the HMGA1 results, overexpression of GRP75 also increased the phosphorylation of both JNK and c-JUN in LUAD cells, and the upregulation of phosphor-JNK and phosphor-c-JUN levels induced by GRP75 overexpression was blocked by silencing HMGA1 (Fig. 6d).
Notably, treatment with the JNK inhibitor JNK IN8 effectively blocked GRP75- and/or HMGA1-overexpression (Additional file 4: Fig S3) inhibited cell viability (Fig. 6e), migration and invasion (Fig. 6f). Collectively, these results reveal that the GRP75/HMGA1 axis promotes LUAD progression by activating the JNK/c-JUN signaling pathway (Fig. 6g).

**Discussion**

Here, we report for the first time that upregulated expression levels of both GRP75 and HMGA1 are closely correlated with recurrence and a poor prognosis in stage I LUAD patients. In particular, HMGA1 has great potential as an independent biomarker for predicting the recurrence and poor prognosis of stage I LUAD patients. Our results are supported by research from other groups, although not in LUAD, similar results have been obtained in other tumors. According to Yi et al., a high expression level of GRP75 is closely correlated with liver cancer early recurrence[22]. According to Liu et al., high expression of HMGA1 is closely correlated with glioblastoma recurrence[23]. However, the sample size used in this study is limited, so the finding needs to be further verified in a larger clinical sample before clinical use.

Here, we also clarified the mechanism of HMGA1 upregulation in LUAD. GRP75 is an oncogene that is overexpressed in several cancers[24] and involved in posttranslational modification of some transcription factors, especially ubiquitinylation[25]. For instance, GRP75 stabilizes the proproliferative association of Hsp90 and epidermal growth factor receptor by rescuing the Tid1L-promoted ubiquitinylation and degradation of epidermal growth factor receptor, thereby contributing to counteracting death pathways[26]. In this study, we identified that GRP75 was upregulated in stage I LUAD and that overexpression of GRP75 promoted LUAD cell progression. In addition, our in vitro data showed that overexpression of GRP75 upregulated HMGA1 protein levels, GRP75 directly bound to HMGA1, GRP75 prolonged the half-life of the HMGA1 protein and GRP75 inhibited HMGA1 ubiquitination. Notably, the expression level of GRP75 was positively correlated with the HMGA1 expression level in LUAD. Together, these findings suggest that abnormally overexpressed GRP75 contributes to early-stage LUAD progression and causes HMGA1 upregulation in LUAD by inhibiting ubiquitination-mediated HMGA1 degradation via direct binding to HMGA1. However, the detailed mechanism by which GRP75 inhibits HMGA1 ubiquitination and the mechanism underlying GRP75 upregulation in stage I LUAD need further study.

Finally, we elucidated the oncogenic mechanism of GRP75 and HMGA1 in LUAD progression. Clarification of the molecular mechanisms of tumor occurrence and progression is urgently needed to help identify effective therapeutic targets. The oncogenic role and therapeutic potential of GRP75 or HMGA1 have been reported in several cancers[27–29]. According to Resar et al., HMGA1 appears to drive transformation in human lung cancer[30], and inhibition of HMGA1 can dramatically inhibit lung cancer cell growth and migration[31, 32]. Additionally, several studies have indicated that GRP75 stimulates cell growth, proliferation and stemness[33–35] and that inhibiting GRP75 can significantly inhibit cancer progression[36]. Consistently, the present study results also show that both GRP75 and HMGA1 play oncogenic roles in LUAD and exhibit potential as therapeutic targets in LUAD. However, the highlight of
this study is that we demonstrated for the first time that GRP75 depends on HMGA1 to play an oncogenic role in LUAD. Notably, we identified that the GRP75/HMGA1 axis plays an oncogenic role by activating JNK/c-JUN signaling. Various findings have reported that activation of the JNK/c-JUN signaling pathway is closely correlated with cancer progression, including recurrence[37–39]. For instance, Jorgense et al. reported that activation of c-JUN was associated with cell proliferation and a shortened relapse-free period in superficially spreading malignant melanoma[40]. According to Hagiwara et al., activation of c-JUN predicts a high risk of recurrence after hepatic resection for hepatocellular carcinoma[41]. To the best of our knowledge, this is the first evidence that HMGA1, as a critical target gene of GRP75, activates the JNK/c-JUN signaling pathway in the progression mechanism of lung adenocarcinoma. However, the detailed mechanism by which HMGA1 activates the JNK pathway in LUAD requires further study.

Conclusions

Our study combined TCGA dataset analysis and experimental studies to establish the tumorigenic roles of HMGA1 and GRP75 in stage I LUAD and the mechanism of HMGA1 upregulation. We show that GRP75 causes upregulation of HMGA1 expression by inhibiting ubiquitination through binding to HMGA1 in LUAD and that upregulated HMGA1 expression promotes LUAD progression by activating the JNK/c-JUN signaling pathway. Additionally, our findings suggest that both GRP75 and HMGA1 are useful therapeutic targets for LUAD and that a high expression level of HMGA1 is an independent biomarker for predicting recurrence and a poor prognosis in stage I LUAD.

Abbreviations

NSCLC: Non-small-cell lung cancer; LUAD: Lung adenocarcinoma; HMGA1: High-mobility group AT-hook 1; EMT: Epithelial-mesenchymal transition; CSCs: Cancer stem cells; GRP75: Glucose-regulated protein 75-kDa; HSPA9: Heat shock protein family A (Hsp70) member 9; TCGA: The Cancer Genome Atlas; PTM: Post-translational modification; JNKs: c-JUN N-terminal kinases; MAPK: mitogen-activated protein kinase; RFS: recurrence-free survival; OS: overall survival; CCK8: cell counting kit-8; CHX: Cycloheximide.

Declarations

Acknowledgements

Not applicable.

Funding

This work was supported by the National Natural Science Foundation of China (81672283 to H.J.), the Natural Science Foundation of Chong Qing, China (cstc2015jcyjA10073 to S.T.), and the Startup Fund for Talented Scholars of Daping Hospital, Army Medical University (to H.J.).

Availability of data and materials
All data generated of analyzed during this study are included in this published article and its supplementary information files. The datasets generated and used in this study are available from the corresponding author on reasonable request.

**Authors’ contributions**

HJ and QYT contributed to the conception and design of this study. GBQ performed laboratory and animal experiments. GBQ, RTW, SNW, SLT and HJ analyzed the data. HJ and GBQ wrote the manuscript. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

This animal protocols in this study were approved by the Animal Research Committee of Army Medical University.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**References**

1. Herbst RS, Morgensztern D, Boshoff C. The biology and management of non-small cell lung cancer. Nature. 2018;553(7689):446–54.
2. Chen Z, Fillmore CM, Hammerman PS, Kim CF, Wong KK. Non-small-cell lung cancers: a heterogeneous set of diseases. Nat Rev Cancer. 2014;14(8):535–46.
3. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WE, Nicholson AG, Groome P, Mitchell A, Bolejack V, et al. The IASLC Lung Cancer Staging Project: Proposals for Revision of the TNM Stage Groupings in the Forthcoming (Eighth) Edition of the TNM Classification for Lung Cancer. J Thorac Oncol. 2016;11(1):39–51.
4. Yoshizawa A, Motoi N, Riely GJ, Sima CS, Gerald WL, Kris MG, Park BJ, Rusch VW, Travis WD. Impact of proposed IASLC/ATS/ERS classification of lung adenocarcinoma: prognostic subgroups and implications for further revision of staging based on analysis of 514 stage I cases. Mod Pathol. 2011;24(5):653–64.
5. Brock MV, Hooker CM, Ota-Machida E, Han Y, Guo M, Ames S, Glockner S, Piantadosi S, Gabrielson E, Pridham G, et al. DNA methylation markers and early recurrence in stage I lung cancer. N Engl J Med. 2008;358(11):1118–28.
6. Reeves R. Nuclear functions of the HMG proteins. Biochimica et Biophysica Acta (BBA) -. Gene Regulatory Mechanisms. 2010;1799(1):3–14.
7. Sarhadi VK, Wikman H, Salmenkivi K, Kuosma E, Sioris T, Salo J, Karjalainen A, Knuutila S, Anttila S. Increased expression of high mobility group A proteins in lung cancer. J Pathol. 2006;209(2):206–12.
8. Lin S, Peng F. Association of SIRT1 and HMGA1 expression in non-small cell lung cancer. Oncology letters. 2016;11(1):782–8.
9. Wang Y, Hu L, Zheng Y, Guo L. HMGA1 in cancer: Cancer classification by location. J Cell Mol Med 2019.
10. Cao XP, Cao Y, Zhao H, Yin J, Hou P. HMGA1 promoting gastric cancer oncogenic and glycolytic phenotypes by regulating c-myc expression. Biochem Biophys Res Commun. 2019;516(2):457–65.
11. Narita M, Narita M, Krizhanovsky V, Nunez S, Chicas A, Hearn SA, Myers MP, Lowe SW. A novel role for high-mobility group A proteins in cellular senescence and heterochromatin formation. Cell. 2006;126(3):503–14.
12. Xian L, Georgess D, Huso T, Cope L, Belton A, Chang Y-T, Kuang W, Gu Q, Zhang X, Senger S, et al: HMGA1 amplifies Wnt signalling and expands the intestinal stem cell compartment and Paneth cell niche. Nature communications 2017, 8.
13. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.
14. Moody S, Perez D, Pan T, Sarkisian C, Portocarrero C, Sterner C, Notorfrancesco K, Cardiff R, Chodosh L. The transcriptional repressor Snail promotes mammary tumor recurrence. Cancer cell. 2005;8(3):197–209.
15. MacDonagh L, Gray SG, Breen E, Cuffe S, Finn SP, O'Byrne KJ, Barr MP. Lung cancer stem cells: The root of resistance. Cancer letters. 2016;372(2):147–56.
16. Bocci F, Gearhart-Serna L, Boareto M, Ribeiro M, Ben-Jacob E, Devi GR, Levine H, Onuchic JN, Jolly MK. Toward understanding cancer stem cell heterogeneity in the tumor microenvironment. Proc Natl Acad Sci USA. 2019;116(1):148–57.
17. Zhang Y, Xu W, Guo H, Zhang Y, He Y, Lee SH, Song X, Li X, Guo Y, Zhao Y, et al. NOTCH1 Signaling Regulates Self-Renewal and Platinum Chemoresistance of Cancer Stem–like Cells in Human Non–Small Cell Lung Cancer. Cancer research. 2017;77(11):3082–91.
18. Valent P, Bonnet D, De Maria R, Lapidot T, Copland M, Melo JV, Chomienne C, Ishikawa F, Schuringa JJ, Stassi G, et al. Cancer stem cell definitions and terminology: the devil is in the details. Nat Rev Cancer. 2012;12(11):767–75.
19. Alamgeer M, Ganju V, Szczepny A, Russell PA, Prodanovic Z, Kumar B, Wainer Z, Brown T, Schneider-Kolsky M, Conron M, et al. The prognostic significance of aldehyde dehydrogenase 1A1 (ALDH1A1) and CD133 expression in early stage non-small cell lung cancer. Thorax. 2013;68(12):1095–104.
20. Li T, Fan J, Wang B, Traugh N, Chen Q, Liu JS, Li B, Liu XS. TIMER: A Web Server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells. Cancer research. 2017;77(21):e108–10.
21. Xu CX, Jere D, Jin H, Chang SH, Chung YS, Shin JY, Kim JE, Park SJ, Lee YH, Chae CH, et al. Poly(ester amine)-mediated, aerosol-delivered Akt1 small interfering RNA suppresses lung tumorigenesis. Am J Respir Crit Care Med. 2008;178(1):60–73.
22. Yi X, Luk JM, Lee NP, Peng J, Leng X, Guan XY, Lau GK, Beretta L, Fan ST. Association of mortalin (HSPA9) with liver cancer metastasis and prediction for early tumor recurrence. Mol Cell Proteomics. 2008;7(2):315–25.

23. Liu B, Pang B, Liu H, Arakawa Y, Zhang R, Feng B, Zhong P, Murata D, Fan H, Xin T, et al. High mobility group A1 expression shows negative correlation with recurrence time in patients with glioblastoma multiforme. Pathol Res Pract. 2015;211(8):596–600.

24. Wadhwa R, Takano S, Kaur K, Deocaris CC, Pereira-Smith OM, Reddel RR, Kaul SC. Upregulation of mortalin/mthsp70/Grp75 contributes to human carcinogenesis. International journal of cancer. 2006;118(12):2973–80.

25. Sane S, Abdullah A, Boudreau DA, Autenried RK, Gupta BK, Wang X, Wang H, Schlenker EH, Zhang D, Telleria C, et al. Ubiquitin-like (UBX)-domain-containing protein, UBXN2A, promotes cell death by interfering with the p53-Mortalin interactions in colon cancer cells. Cell death disease. 2014;5:e1118.

26. Srivastava S, Vishwananathan V, Birje A, Sinha D, D'Silva P. Evolving paradigms on the interplay of mitochondrial Hsp70 chaperone system in cell survival and senescence. Crit Rev Biochem Mol Biol. 2019;54(6):517–36.

27. Xu M, Jin T, Chen L, Zhang X, Zhu G, Wang Q, Lin Z. Mortalin is a distinct bio-marker and prognostic factor in serous ovarian carcinoma. Gene. 2019;696:63–71.

28. Starenki D, Sosonkina N, Hong SK, Lloyd RV, Park JI. Mortalin (GRP75/HSPA9) Promotes Survival and Proliferation of Thyroid Carcinoma Cells. Int J Mol Sci 2019, 20(9).

29. Meireles Da Costa N, Ribeiro Pinto LF, Nasciutti LE, Palumbo A Jr: The Prominent Role of HMGA Proteins in the Early Management of Gastrointestinal Cancers. Biomed Res Int 2019, 2019:2059516.

30. Hillion J, Wood LJ, Mukherjee M, Bhattacharya R, Di Cello F, Kowalski J, Elbahloul O, Segal J, Poirier J, Rudin CM, et al. Upregulation of MMP-2 by HMGA1 Promotes Transformation in Undifferentiated, Large-Cell Lung Cancer. Mol Cancer Res. 2009;7(11):1803–12.

31. Ma Y, Li X, Chen S, Du B, Li Y. MicroRNA-4458 suppresses migration and epithelial-mesenchymal transition via targeting HMGA1 in non-small-cell lung cancer cells. Cancer Manag Res. 2019;11:637–49.

32. Panneerselvam J, Srivastava A, Muralidharan R, Wang Q, Zheng W, Zhao L, Chen A, Zhao YD, Munshi A, Ramesh R. IL-24 modulates the high mobility group (HMG) A1/miR222/AKT signaling in lung cancer cells. Oncotarget. 2016;7(43):70247–63.

33. Sun J, Che SL, Piao JJ, Xu M, Chen LY, Lin ZH. Mortalin overexpression predicts poor prognosis in early stage of non-small cell lung cancer. Tumour biology: the journal of the International Society for Oncodevelopmental Biology Medicine. 2017;39(3):1010428317695918.

34. Yun CO, Bhargava P, Na Y, Lee JS, Ryu J, Kaul SC, Wadhwa R. Relevance of mortalin to cancer cell stemness and cancer therapy. Scientific reports. 2017;7:42016.

35. Na Y, Kaul SC, Ryu J, Lee JS, Ahn HM, Kaul Z, Kalra RS, Li L, Widodo N, Yun CO, et al. Stress chaperone mortalin contributes to epithelial-mesenchymal transition and cancer metastasis. Cancer research. 2016;76(9):2754–65.
36. Lu WJ, Lee NP, Kaul SC, Lan F, Poon RT, Wadhwa R, Luk JM. Mortalin-p53 interaction in cancer cells is stress dependent and constitutes a selective target for cancer therapy. Cell Death Differ. 2011;18(6):1046–56.

37. Lee S, Rauch J, Kolch W. Targeting MAPK Signaling in Cancer: Mechanisms of Drug Resistance and Sensitivity. Int J Mol Sci 2020, 21(3).

38. Hoang VT, Ny Swaner K, Torres-Ayuso P, Brognard J. The protein kinase MAP3K19 phosphorylates MAP2Ks and thereby activates ERK and JNK kinases and increases viability of KRAS-mutant lung cancer cells. J Biol Chem 2020.

39. Zhou C, Huang Y, Chen Y, Xie Y, Wen H, Tan W, Wang C. miR-602 Mediates the RASSF1A/JNK Pathway, Thereby Promoting Postoperative Recurrence in Nude Mice with Liver Cancer. OncoTargets therapy. 2020;13:6767–76.

40. Jorgensen K, Davidson B, Florenes VA. Activation of c-jun N-terminal kinase is associated with cell proliferation and shorter relapse-free period in superficial spreading malignant melanoma. Modern Pathol. 2006;19(11):1446–55.

41. Hagiwara S, Kudo M, Chung H, Ueshima K, Inoue T, Haji S, Watanabe T, Park A, Munakata H, Sakurai T. Activation of c-Jun N-terminal kinase in non-cancerous liver tissue predicts a high risk of recurrence after hepatic resection for hepatocellular carcinoma. Hepatology research: the official journal of the Japan Society of Hepatology. 2012;42(4):394–400.

**Figures**
Figure 1

HMGA1 expression level was associated with recurrence and poor prognosis in Stage I LUAD. a HMGA1 mRNA expression in different cancers from The Cancer Genome Atlas (TCGA) datasets. TPM, Transcripts Per Million. b Expression of HMGA1 in all stage LUAD tumor samples compared with normal tissues. c Expression level of HMGA1 in Stage I LUAD samples significantly higher than normal. d The HMGA1 expression level was significantly associated with recurrence in stage I LUAD patients from the TCGA cohort. e The HMGA1 high-expression group had a significantly lower median time of RFS (Recurrence-free survival) rate than those in the HMGA1 low-expression group in stage I LUAD patients from the TCGA cohort. f The HMGA1 high-expression group had a significantly lower OS (overall survival) rate than those in the HMGA1 low-expression group in stage I LUAD patients from the TCGA cohort. *, p < 0.05; ***, p < 0.001.
Figure 2

Overexpression of HMGA1 increases LUAD cell viability and metastasis. a Western blot analysis of the expression of HMGA1 in A549 and PC9 cells. Indicated cells were transfected with construct that expressing green fluorescent protein (GFP)-tagged HMGA1. After 72 hours of transfection, HMGA1 was detected using HMGA1 antibodies. b Overexpression of HMGA1 significantly stimulated LUAD cell viability using the CCK8 assays. c-d Overexpression of HMGA1 significantly stimulated Soft agar colony formation, migration and invasion of LUAD cells. Three independent experiments were conducted. Data were presented as the mean±S.D. e Weights and images of tumors from PC9 xenograft model (n=5 per group). f HMGA1 overexpression stimulated tumor growth in PC9 xenograft model. g
Immunohistochemistry assay showed that HMGA1 overexpression increased cell proliferation marker protein Ki67-positive LUAD cells. The tumor tissues were from PC9 xenograft model. h-i Overexpression of HMGA1 significantly promoted lung metastases. Images of metastatic lung nodules in nude mice (n=5 per group) and HE staining were performed after four weeks by tail-vein injection of A549 LUAD cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Figure 3**

Inhibition of HMGA1 inhibits LUAD progression. a HMGA1 siRNA treatment significantly inhibited HMGA1 expression in LUAD cells. Indicated cells were transfected with siRNAs of HMGA1. After 72 hours of transfection, cells were subjected to Western blot analysis. b CCK8 assays showed that inhibition of HMGA1 significantly inhibits LUAD cells viability. c-d Inhibition of HMGA1 significantly suppressed Soft agar colony formation, migration and invasion of LUAD cells. Three independent experiments were
conducted. Data were presented as the mean±S.D. e Weight and image of tumors showed that inhibition of HMGA1 significantly inhibited tumor size in PC9 xenograft model (n=5 per group). f The tumor volume-time curves had shown that HMGA1 down-expression inhibited tumor growth. g Immunohistochemistry assay showed that inhibition of HMGA1 significantly decreased Ki67-positive cells in LUAD tissues from PC9 xenograft model. h-i HMGA1 down-expression inhibited lung metastases. Images of metastatic lung nodules in nude mice (n=5 per group) and HE staining were performed after four weeks by tail-vein injection of A549 LUAD cells. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
GRP75 interacts with HMGA1 and plays oncogenic role in LUAD. a Silver staining image of proteins that interact with HMGA1 in LUAD cells. A549 cells were transfected with vector or HMGA1 expression plasmid for 72 hours. Cell lysate was immunoprecipitated with anti-HMGA1 and IgG, and the proteins interact with HMGA1 were separated with SDS-PAGE, and stained with silver. b Venn plot of differential gene expression analysis. The proteins interact with HMGA1 and IgG were detected by mass spectrometry. c Co-IP assays were performed to confirm the interaction of GRP75 with HMGA1 in A549 cells with anti-HMGA1 or anti-GRP75 antibody. d TCGA data set analysis showed that GRP75 protein expression level is positively correlated with HMGA1 protein expression level in stage I LUAD. TPM, Transcripts Per Million. e TCGA data set analysis showed that expression of GRP75 was significantly increased in stage I LUAD samples compared with normal tissues. f TCGA data set analysis showed the expression level of GRP75 was closely associated with recurrence in stage I LUAD patients. g TCGA data set analysis showed the expression level of GRP75 was closely associated with shorter recurrence-free survival time in stage I LUAD patients. h CCK8 analysis showed that GRP75 stimulated LUAD cells viability. i Transwell assays showed that GRP75 stimulated migration and invasion of LUAD cells. j CCK8 assay showed that inhibition of GRP75 significantly inhibits LUAD cells viability. k Inhibition of GRP75 significantly suppressed migration and invasion of LUAD cells. Three independent experiments were conducted. Data were presented as the mean±S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 5

HMGA1 is the functional downstream target of GRP75 in LUAD cells. a GRP75 positively regulated HMGA1 expression in LUAD cells, but HMGA1 did not affect GRP75 expression. Indicated cells were transfected with indicated plasmids or siRNA for 72 hours, then subjected to Western blot analysis. Bands corresponding to RFP-GRP75 fusion protein runs with apparent molecular weights of about 100 kD. b CCK8 assay showed that silencing of HMGA1 blocked overexpression of GRP75-induced LUAD cell viability stimulation. c Transwell assay showed that silencing of HMGA1 blocked overexpression of GRP75-induced stimulation of migration and invasion. d TCGA data set analysis showed there was no
correlation between the expression level of GRP75 and recurrence-free survival (RFS) time in stage I LUAD patients with low expression of HMGA1. e GRP75 did not affect HMGA1 mRNA expression in LUAD cells. Indicated cells were transfected with indicated plasmid or siRNA for 72 hours, then cells were subjected to qRT-PCR analysis. f Overexpression of GRP75 significantly prolonged the half-life time of HMGA1 in LUAD cells. A549 cells with or without overexpressing GRP75 were treated with CHX (100 µg/ml, CST) for the indicated times. Western blot analysis and Grayscale analysis of HMGA1 protein levels were showed. g Overexpression of GRP75 inhibited HMGA1 ubiquitination in LUAD cells. A549 cells were transfected with GRP75 plasmids for 72 hours, then cells were treated with 20μM MG132 for 8 hours. The whole-cell lysates were subjected to immunoprecipitation with HMGA1 antibody and Western blot with anti-Ub antibody to detect ubiquitylated HMGA1. Three independent experiments were conducted. Data were presented as the mean±S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Figure 6

GRP75/HMGA1 axis stimulates LUAD progression by activating JNK/c-JUN signaling pathway.  

a Heatmap shown genes that expression level was affected by overexpressing HMGA1 or GRP75 in LUAD cells. A549 cells were transfected with indicated plasmid for 72 hours, then subjected to mRNA sequencing. b MAPK signaling was significantly affected by GRP75/HMGA1 axis in LUAD cells. KEGG signal pathway enrichment analysis was performed using genes that similarly affected by HMGA1 and
GRP75 overexpression. c Western blot analysis showed that HMGA1 positively regulated the phosphorylation of JNK(Thr183/Tyr185), c-JUN (Ser63) and c-JUN (Ser73) in A549 cells. Cells were transfected with HMGA1 plasmids or HMGA1 siRNAs for 72 hours, then subjected to Western blot analysis. d Inhibition of HMGA1 blocked GRP75 overexpression induced phosphorylation of JNK and c-JUN in A549 cells. Cells were transfected with HMGA1 plasmids or HMGA1 siRNAs for 72 hours, then subjected to Western blot analysis. e CCK8 analysis showed that JNK inhibitor treatment blocked overexpression HMGA1 or/and GRP75-induced cell viability stimulation. f Transwell analysis showed that JNK inhibitor treatment blocked overexpression of HMGA1 or/and GRP75-induced stimulation of cell migration and invasion. Indicated cells were transfected with indicated plasmid for 48 hours, then cells were treated with 1μM JNK IN8 (JNK inhibitor) for 24 hours. g A schematic model for the progression mechanism of LUAD by GRP75/HMGA1 axis in LUAD cells. Three independent experiments were conducted. Data were presented as the mean±S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1TableS1bs.pdf
- Additionalfile3FigS2bs.pdf
- Additionalfile4FigS3bs.pdf
- Additionalfile2FigS1bs.pdf