

HuR affects chemoresistance of small cell lung cancer by regulating FGFR1 expression

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Abstract. Human antigen R (HuR), an RNA-binding protein, has been demonstrated to serve an oncogenic role in various types of cancer. Fibroblast growth factor receptor-like 1 (FGFR1) has been shown to regulate small cell lung cancer (SCLC) chemoresistance. In the present study, the role of HuR in chemoresistance of SCLC, as well as its possible molecular mechanism involving FGFR1, was explored by reverse transcription-quantitative PCR, western blotting, Cell Counting Kit-8 assay, flow cytometry and RNA immunoprecipitation. The results revealed that HuR expression levels were markedly upregulated in drug-resistant SCLC cell lines (H69AR and H446DDP) compared with in the parental cell lines (H69 and H446). Knockdown of HuR in drug-resistant SCLC cells enhanced drug sensitivity, cell apoptosis and cell cycle arrest. Furthermore, molecular mechanism studies indicated that HuR could bind and regulate FGFR1 expression levels to increase FGFR1 mRNA stability. Taken together, the present study suggested that HuR may mediate chemoresistance of SCLC by regulating FGFR1 expression. HuR may represent a prognostic predictor and a potential target for overcoming chemoresistance in SCLC.

Introduction

Small cell lung cancer (SCLC) is a malignant tumor of neuroendocrine origin, accounting for ~15% of all cases of lung cancer worldwide. Although it is a type of lung cancer, it is different from other forms of lung cancer with regard to its pathology, molecular basis and clinical treatment (1,2).

First-line treatment of SCLC usually consists of 4-6 cycles of chemotherapy with platinum and etoposide (VP16), and this chemotherapy regimen has not been changed in the past three decades. Patients have a higher initial response to this chemotherapy regimen compared with other treatment strategies, but a number of them rapidly relapse and exhibit chemotherapy resistance after 6 months (3). Therefore, it is important to elucidate the mechanisms of chemoresistance in SCLC.

RNA-binding proteins (RBPs) can bind to coding or non-coding RNAs to regulate their fate, such as stabilization, splicing and localization (4,5). Therefore, RBPs have crucial roles in various cellular processes, including RNA stabilization, splicing and localization. The RBP human antigen R (HuR) is a member of the embryonic lethal abnormal vision (ELAV) gene family and is also known as ELAV-like RNA-binding protein 1 (ELAV1) (6,7). HuR regulates the stability and translation of target mRNAs by binding to the AU-rich elements domain in the 3'-untranslated region (UTR) (8). Lee *et al* (9) reported that HuR promotes cellular senescence via post-transcriptional control of TIN2 expression. Xiao *et al* (10) demonstrated that HuR regulates paneth cell function by post-transcriptional regulation of CNPY3 expression. Zhang *et al* (11) revealed that HuR promotes cell growth by binding to the mRNA of CDK3 in breast cancer cells. Additionally, HuR can bind and affect non-coding RNA functions (12-15). Lan *et al* (14) indicated that long noncoding RNA (lncRNA) OCC-1 can suppress colorectal cancer cell proliferation by binding and destabilizing HuR. Zou *et al* (15) also revealed that lncRNA 00324 can combine with HuR to promote gastric cancer cell proliferation. However, to the best of our knowledge, the role of HuR in SCLC has not been reported. The present study aimed to explore the potential involvement of HuR in SCLC.

Materials and methods

Cell culture and reagents. Human SCLC cells lines NCI-H69, NCI-H446 and NCI-H69AR were purchased from American Type Culture Collection. Cisplatin (DDP)-resistant NCI-H446DDP cells were established by exposing H446 cells to DDP, as previously described (16). All cells were cultured in RPMI-1640 medium (Cytiva) containing 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibiotics (100 mg/ml penicillin and 100 mg/ml streptomycin; Beyotime

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Institute of Biotechnology) in an incubator at 37°C with 5% CO₂. The following antibodies were used: Anti-fibroblast growth factor receptor-like 1 (FGFRL1; cat. no. ab95940) and anti-HuR (cat. no. ab200342) (both from Abcam); and anti-GAPDH (cat. no. BS72410; Bioworld Technology, Inc.).

mRNA sequencing. The cells (H69 and H69AR) were lysed using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and the RNA concentration was measured using NanoDrop 2000 (NanoDrop; Thermo Fisher Scientific, Inc.). mRNA sequencing assays were performed using the BGISEQ-500 platform by BGI Genomics (17).

RNA isolation and RT-quantitative PCR (qPCR). Total RNA was extracted from H69, H69AR, H446 and H446DDP cells using TRIzol reagent. The RNA concentrations were measured using NanoDrop 2000 (NanoDrop; Thermo Fisher Scientific, Inc.). For cDNA synthesis, RT reactions were performed using PrimeScript RT reagent Kit (Tiagen Biotech Co., Ltd.) according to the manufacturer's protocol. Subsequently, qPCR was conducted in an ABI 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using 2X Talent qPCR PreMix (Tiagen Biotech Co., Ltd.). RT-qPCR reactions were performed as follows: 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. GAPDH was used as an endogenous control. The relative mRNA expression levels of target genes were quantified using the 2^{-ΔΔC_q} method (18). The sequences of the primers (Sangon Biotech Co., Ltd.) are shown in Table SI.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and protein concentrations were measured using the BCA Protein Quantitation Kit (CoWin Biosciences). Subsequently, 30 μg protein lysates were separated by SDS-PAGE on 12% gels (Hangzhou Fude Biological Technology, Co. Ltd.) and transferred onto polyvinylidene difluoride membranes (MilliporeSigma). The membranes were then blocked with 5% skim milk (Hangzhou Fude Biological Technology, Co. Ltd.) for 1 h at room temperature, and incubated with specific primary antibodies overnight at 4°C: Anti-FGFRL1 (1:1,000), anti-HuR (1:1,000) and anti-GAPDH (1:1,000). Subsequently, the membranes were incubated with an AP-conjugated anti-rabbit secondary antibody (1:2,000; cat. no. E030220-02; EarthOx Life Sciences) for 1 h at room temperature. The immune complexes were detected using ECL developer solution (Fude Biological Technology, Co., Ltd.) and an imaging system (Bio-Rad Laboratories, Inc.).

Transfection. A total of 1x10⁵ cells (H69AR and H446DDP) were seeded into 6-well plates and allowed to grow until the confluence reached 80%. Based on the manufacturer's instructions, cells were transiently transfected with 0.5 μg small interfering RNAs (siRNAs) against HuR (siHuR) or scrambled siRNA negative control (Shanghai GenePharma Co., Ltd.) using 10 μl Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) and OPTI-MEM (Invitrogen; Thermo Fisher Scientific, Inc.). In the mock group cells, only 10 μl Lipofectamine 3000 and OPTI-MEM were added. For transfection, cells were cultured for 4 h with the Lipofectamine/siRNA mix at 37°C.

After 48 h, the cells were harvested for subsequent experiments. The sequences of siHuR are listed in Table SII.

Drug resistance assay. The cells were transiently transfected and then treated with different concentrations of chemotherapy drugs for 24 h. The chemotherapy drugs used were as follows: Adriamycin (ADM) (Zhejiang Haizheng Pharmaceutical Co., Ltd.), DDP (Shandong Luoxin Pharmaceutical Group Co., Ltd.) or VP16 (Jiangsu Hengrui Pharmaceuticals Co., Ltd.) to determine IC₅₀ values. Finally, 1x10³ cells were incubated with 10 μl Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories, Inc.) for 4 h and absorbance was detected at 450 nm. The optical density value of each well was used to calculate the IC₅₀ of the corresponding chemotherapeutic drug.

Flow cytometric analysis. Transfected cells were seeded in 6-well plates and treated with half the IC₅₀ dose of chemotherapeutic drugs (ADM, DDP and VP16). After 24 h, all cells were collected for further analysis. For apoptosis assays, cells were incubated with Annexin V-APC/PI (cat. no. 88-8007-74; eBioscience; Thermo Fisher Scientific, Inc.) or Annexin V-FITC/PI (CoWin Biosciences) according to the manufacturer's protocols. For cell cycle assays, 1x10⁵ cells/100 ul were fixed with 75% ethanol at 4°C for 12 h. Subsequently, the cells were stained with 50 μl RNaseA and 450 μl PI (MilliporeSigma) in the dark for 30 min at room temperature. All samples were analyzed with a BD FACSVerse flow cytometer (BD Biosciences). The apoptotic rate was calculated using FlowJo 7.6.1 software (FlowJo LLC) and the cell cycle distribution was obtained using ModFit 3.2 software (Verity Software House).

Bioinformatics analysis. The interaction between HuR and FGFRL1 mRNA was predicted by Starbase v2.0 database (<http://starbase.sysu.edu.cn/>).

RNA immunoprecipitation (RIP) assay. RIP was performed using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (cat. no. 17-700; MilliporeSigma) following the manufacturer's protocol. The cells were harvested and lysed using RIP lysis buffer. Anti-HuR or normal rabbit IgG (cat. no. ab172730; Abcam) antibodies were pre-incubated with magnetic beads to form a complex, and cell lysates were then incubated with the magnetic bead-antibody complex overnight at 4°C. Subsequently, the RNA in the immunoprecipitates was purified according to the kit protocol. The immunoprecipitated RNA was extracted with TRIzol and analyzed by RT-qPCR.

mRNA stability assays. The expression of HuR was knocked down by transfection with siHuR for 48 h. The *de novo* RNA synthesis was blocked with 3 mg/ml actinomycin-D (ActD) (Apexbio Technology LLC) at 37°C for 0, 4, 8, 12, 16, 20 and 24 h. Total RNA was harvested at the specified time points (0, 4, 8, 12, 16, 20 and 24 h) and FGFRL1 mRNA expression was measured by RT-qPCR. The half-life of FGFRL1 mRNA was determined by comparing to the mRNA levels detected prior to the addition of ActD.

Statistical analysis. All experiments were performed independently three times under the same conditions. SPSS 20.0 (IBM Corp.) was used for statistical analysis. The results are

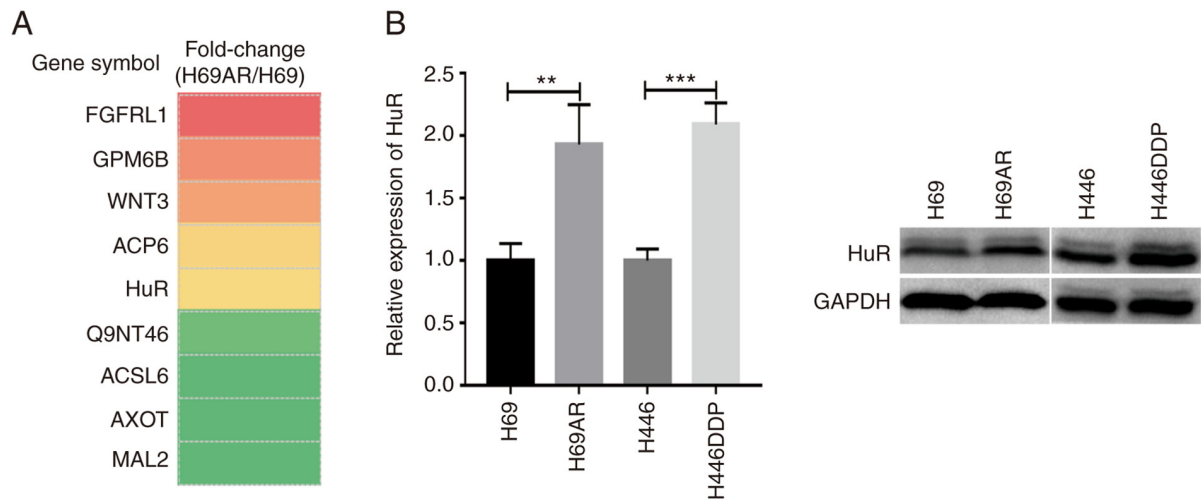


Figure 1. HuR expression is increased in chemoresistant SCLC cells. (A) Analysis of differentially expressed genes in H69 and H69AR cells by high-throughput sequencing. (B) Reverse transcription-quantitative PCR and western blotting showed that HuR levels were increased in chemoresistant SCLC cells. ** $P < 0.01$; *** $P < 0.001$. HuR, human antigen R; SCLC, small cell lung cancer.

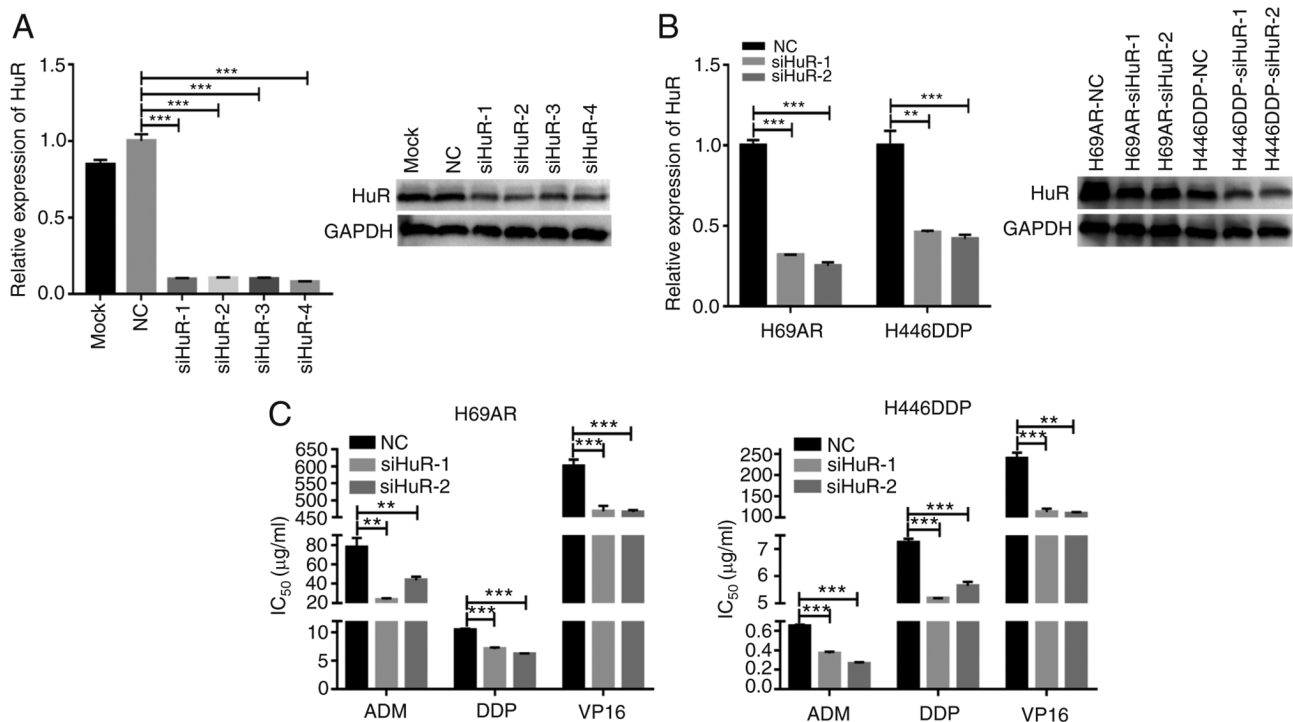


Figure 2. HuR affects the chemoresistance of small cell lung cancer cells. (A) Knockdown of HuR by siRNA was confirmed by reverse transcription-quantitative PCR and western blotting in H69AR cells (B) Knockdown of HuR by siRNA in H69AR and H446DDP cells. (C) IC_{50} values were determined by Cell Counting Kit-8 assay when siHuR-transfected cells were exposed to chemotherapy drugs. ** $P < 0.01$; *** $P < 0.001$. HuR, human antigen R; siRNA, small interfering RNA; NC, negative control; ADM, adriamycin; DDP, cisplatin; VP16, etoposide.

presented as the mean \pm standard deviation. Statistical differences were analyzed by independent-sample t-tests or one-way analysis of variance followed by Bonferroni correction to compare differences between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

HuR expression is increased in chemoresistant SCLC cells. Differentially expressed genes between chemoresistant

and chemotherapy-sensitive SCLC cells were screened by high-throughput sequencing (Fig. 1A). The expression levels of HuR were high in chemoresistant SCLC cells. To further verify the results of sequencing, HuR expression was measured by RT-qPCR and western blot analysis in H69/H69AR and H446/H446DDP cells. The results demonstrated that the expression levels of HuR were increased in multidrug-resistant SCLC cells (H69AR and H446DDP) compared with those in the parental cells (H69 and H446) (Fig. 1B). These results suggested that HuR expression levels were upregulated in chemoresistant SCLC cells.

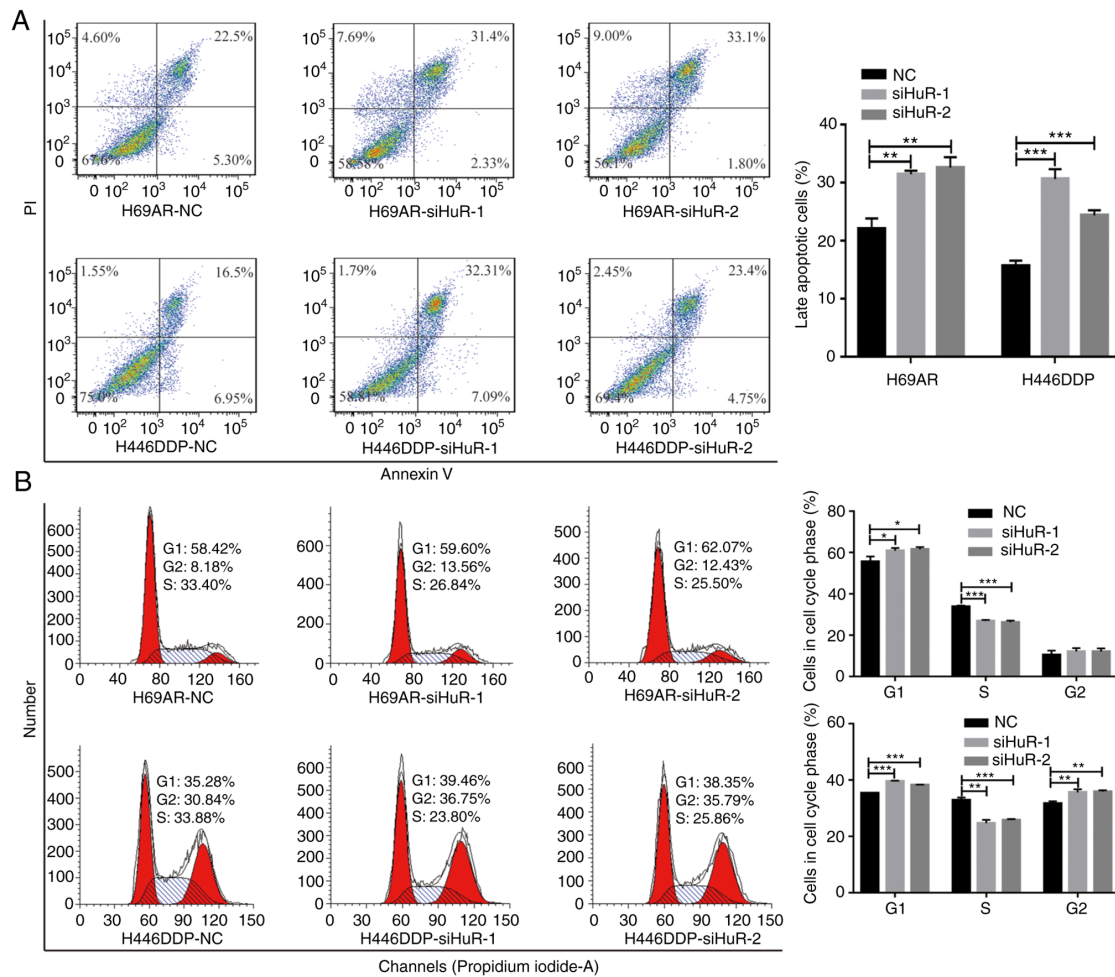


Figure 3. Knockdown of HuR increases cell sensitivity to chemotherapy drugs by enhancing cell apoptosis and cell cycle arrest. (A) Cell apoptosis and (B) cell cycle progression was evaluated by flow cytometric analysis in siHuR-transfected small cell lung cancer cells following adriamycin exposure. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. HuR, human antigen R; si, small interfering; NC, negative control.

Knockdown of HuR increases cell sensitivity to chemotherapeutic drugs. To determine whether HuR can regulate SCLC chemotherapy resistance, four siRNAs (Table SII) were designed to transfect H69AR cells. RT-qPCR showed that HuR expression was slightly lower in cells transfected with siHuR-1 and siHuR-4 compared with siHuR-2 and siHuR-3, whereas western blot analysis revealed that HuR expression was slightly lower in cells transfected with siHuR-1 and siHuR-2 compared with siHuR-2 and siHuR-4 (Fig. 2A). Therefore, siHuR-1 and siHuR-2 were selected for the subsequent experiments. Notably, the expression levels of HuR were reduced in H69AR and H446DDP cells transfected with siHuR compared with those in the NC groups (Fig. 2B). Subsequently, SCLC cells were treated with various chemotherapeutic drugs (ADM, DDP and VP16). CCK-8 assays revealed that the IC_{50} values were markedly decreased following knockdown of HuR in H69AR and H446DDP cells (Fig. 2C). These experiments confirmed that HuR could regulate the chemoresistance of SCLC.

Knockdown of HuR increases cell sensitivity to chemotherapy drugs by enhancing cell apoptosis and cell cycle arrest. Flow cytometric analysis demonstrated that knockdown of HuR in H69AR and H446DDP cells increased cell apoptosis (Fig. 3A,

S1A and S2A) and cell cycle arrest (Fig. 3B, S1B and S2B) following drug exposure.

HuR regulates FGFR1 expression by binding and stabilizing FGFR1 mRNA in SCLC cells. The present study indicated that HuR was involved in the chemoresistance of SCLC. To further investigate its possible regulatory mechanism, the binding sites of HuR were analyzed using the StarBase v2.0 database. The results revealed that HuR has four potential binding sites on FGFR1 mRNA in six different tumors (breast cancer, colon and rectal adenocarcinoma, head and neck squamous cell carcinoma, chromophobe renal cell carcinoma, ovarian serous cystadenocarcinoma, and papillary thyroid carcinoma). Our previous study confirmed that FGFR1 can regulate SCLC chemoresistance (19). RIP assays revealed that HuR interacted with FGFR1 mRNA in H69AR cells (Fig. 4A). Therefore, the present study assessed whether HuR could regulate the expression of FGFR1 in chemoresistant SCLC cells. As expected, knockdown of HuR decreased the mRNA and protein expression levels of FGFR1 (Fig. 4B). HuR is an RBP that serves an important role in the regulation of mRNA stability (6-8); therefore, it was hypothesized that HuR may affect FGFR1 expression by regulating FGFR1 mRNA stability in SCLC cells.

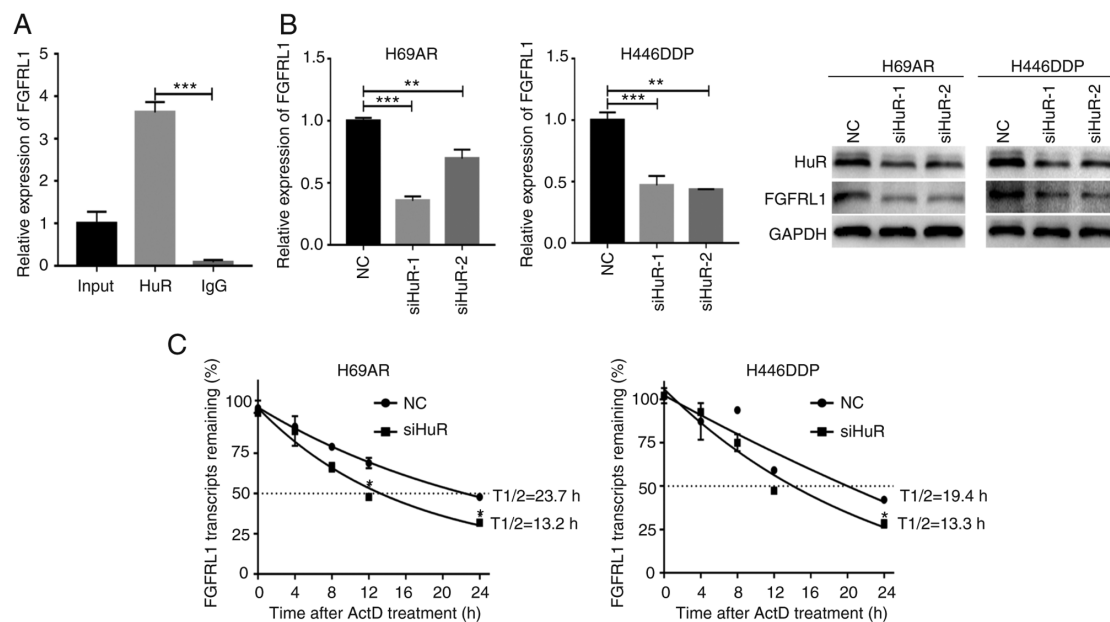


Figure 4. HuR regulates FGFR1 expression by binding and stabilizing FGFR1 mRNA. (A) RT-qPCR was used to evaluate the abundance of HuR mRNA in the HuR-IP and IgG-IP groups following RNA IP. (B) mRNA and protein expression levels of FGFR1 were measured in siHuR-transfected cells. (C) After HuR knockdown in H69AR and H446DDP cells, the half-life of FGFR1 mRNA was detected by RT-qPCR. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as indicated or vs. NC. HuR, human antigen R; si, small interfering; NC, negative control; FGFR1, fibroblast growth factor receptor-like 1; ActD, actinomycin-D; RT-qPCR, reverse transcription quantitative PCR; IP, immunoprecipitation; T1/2, half-life time.

Notably, the decay rate of FGFR1 mRNA was faster in siHuR-transfected cells compared with that in the control group following treatment with ActD, a transcriptional inhibitor (Fig. 4C). These results suggested that HuR can regulate the expression of FGFR1 by interacting with its mRNA to increase the mRNA stability.

Discussion

In recent years, a number of studies have revealed the crucial roles of HuR in the development and progression of numerous diseases, including cancer (20–22). HuR has also been reported to regulate cell growth, migration and invasion in non-SCLC (23); however, to the best of our knowledge, the role of HuR in SCLC remains unknown. The present study identified that HuR expression was increased in chemoresistant SCLC cells compared with in chemosensitive parental cells. Functional experiments revealed that knockdown of HuR could weaken chemotherapy resistance. To the best of our knowledge, the present study is the first to investigate the role of HuR in SCLC.

There are two main mechanisms underlying post-transcriptional modification of mRNA: i) Small non-coding microRNAs bind to mRNA 3'-UTR leading to its destabilization and degradation; ii) RBPs bind to mRNA AU-rich elements in the 3'-UTR or introns resulting in stabilization and translation (24–26). HuR, an RBP, has been reported to be related to tumor occurrence, development and metastasis in various types of cancer, such as breast, lung and colorectal cancer (27–29). To explore whether HuR could act as a post-transcriptional modification protein in SCLC chemoresistance, bioinformatics analysis was conducted to identify its potential interactions with genes. The results showed four potential binding sites between HuR and FGFR1 mRNA, and downregulation of

HuR was shown to reduce the expression levels of FGFR1 in chemotherapy-resistant SCLC cells. In addition, RIP and mRNA stability assays confirmed that HuR interacted with FGFR1 mRNA to increase its stability. Future experiments will evaluate the role of HuR in *in vivo* models.

FGFR1 is a member of the FGFR family, but lacks the classical kinase domain (30). Our previous research confirmed that FGFR1 affected the chemoresistance of SCLC by modulating the PI3K/Akt pathway via ENO1 (19). The present study explored the upstream regulatory mechanism of FGFR1 and revealed that the RBP HuR could affect its expression by enhancing FGFR1 mRNA stability. Taken together, the present study confirmed that HuR promoted SCLC chemoresistance by regulating FGFR1 expression. HuR is an RBP encoded by the ELAV1 gene that acts by stabilizing mRNA and regulating gene expression (8). Whether it functions via other chemoresistance mechanisms in SCLC requires further exploration. The present study indicated that HuR may be a vital predictor and a potential therapeutic target for SCLC diagnosis and therapy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable

request. The sequencing datasets generated and/or analyzed during the current study are available in the Sequence Read Archive repository, under accession number PRJNA836624 (<https://www.ncbi.nlm.nih.gov/sra/SRX15216270>).

Authors' contributions

XHD, RC and LLG conceived and designed the study. RC, XHD and DSL performed the experiments. RC, DSL and AHL analyzed the data. XHD, RC and LLG wrote the manuscript. XHD and LLG confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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