Human antigen R affects the migration and invasion of human lung cancer A549 cells and regulates E-cadherin suppressor Snail

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INTRODUCTION

Although substantial progress was achieved in the understanding of lung cancer genetics, etiology, risk factors, management and treatments, the incidence of lung cancer is still rising throughout the world, causing the highest number of deaths (1.8 million) among all cancers (1-3). The cancer statistics 2021 showed that in the United States nearly a quarter of cancer-related mortality was attributed to lung cancer, which was 2.0-fold and 1.47-fold higher than the second-highest cancer deaths in men (prostate cancer) and women (breast cancer), respectively (4). One of the reasons behind this high mortality rate is that lung cancer is usually diagnosed at advanced metastatic stages, in which the migration of tumor cells plays an initial and critical role (5). Thus, targeting the migration of lung cancer cells at early stages is believed to be a promising therapeutic strategy to improve the prognosis and survival of patients (6).

The migration of cancer cells involves a number of pathophysiological molecular signatures, among which epithelial cadherin (E-cadherin), a well-characterized tumor-suppressor, draws a significant amount of attention (7). E-cadherin is a single-pass transmembrane glycoprotein mediating the interactions and connections between the epithelial cells at adherens junctions. Because many solid tumors originated from the epithelial tissue, a coprotein mediating the interactions and connections between the epithelial cells at adherens junctions. Because many solid tumors originated from the epithelial tissue, a coprotein mediating the interactions and connections between the epithelial cells at adherens junctions. Because many solid tumors originated from the epithelial tissue, a coprotein mediating the interactions and connections between the epithelial cells at adherens junctions. Because many solid tumors originated from the epithelial tissue, a coprotein mediating the interactions and connections between the epithelial cells at adherens junctions.
The expression of E-cadherin can be modulated at multiple levels, including transcription regulations via repressors and promoter hypermethylation, post-transcriptional regulations via RNA-binding proteins (RBPs) and non-coding RNAs, and other epigenetic modifications (12). In the characterized repressors of E-cadherin, Snail, a key zinc finger transcriptional factor, is suggested to serve as a convergence bridge in a myriad of molecules controlling cell movement and the epithelial-mesenchymal transition (EMT), featured by alterations in E-cadherin and N-cadherin expressions (13). Recently, a ubiquitously expressed post-transcriptional RBP human antigen R (HuR) was found to affect E-cadherin expression and EMT in cigarette smoke extract-treated human airway epithelial cells (14,15). In addition, HuR was demonstrated to influence the expression of E-cadherin in esophageal squamous cell carcinoma and human colon carcinoma cells (16-18). As an RNA stabilizing protein, HuR was also shown to impact on Snail expressions in gastric cancer and pancreatic cancer cells (19,20). However, whether HuR could directly stabilize Snail to affect EMT marker expression in lung cancer cells is currently not documented.

Despite that many previous studies have reported that a wide variety of factors could influence the progression, metastasis and stemness of lung cancer cells via regulating HuR expression (21-27), and a number of nanoparticle-based drug delivery approaches involving or targeting HuR were proposed to treat lung cancer (28), reports regarding the direct effect of HuR on the migration of lung cancer cells as well as the mechanistic regulation by E-cadherin, N-cadherin and Snail is absent. Accordingly, the aim of this study was to investigate whether the knockdown and overexpression of HuR could directly interfere with the migration and invasion of adenocarcinomic human alveolar basal epithelial cells A549 and whether HuR could directly interact with Snail to affect EMT marker expression in lung cancer cells.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS), penicillin-streptomycin, trypsin and RPMI-1640 medium were purchased from HyClone (Logan UT). Anti-E-cadherin, anti-Snail and anti-N-cadherin antibodies were from Cell Signaling Technology (Danvers, MA). Anti-β-actin antibody, vanadyl-ribonucleoside complex (VRCS), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), proteinase K and Protein A sepharose beads (CL-4B) were bought from Sigma Aldrich (MO, USA). The anti-HuR antibody was obtained from Santa Cruz (Santa Cruz, CA). Anti-rabbit IgG and anti-mouse IgG secondary antibodies (horseradish peroxidase) were purchased from Zhongshan Goldenbridge Biotechnology (Beijing, China). RNase inhibitor was acquired from ABeclonal Technology (Wuhan, China). The protease inhibitor cocktail was bought from MCE (NJ, USA). Mouse IgG1 isotype control was purchased from R & D systems (Minneapolis, MN, USA). Trizol, lipofectamine-3000 and yeast tRNA were obtained from Invitrogen (Carlsbad, CA). Enhanced chemiluminescence (ECL) reagent was acquired from EMD Millipore (Billerica, MA). ReverTra Ace qPCR RT master mix kit was bought from Toyobo life science (Osaka, Japan) and the SYBR Green PCR kit was purchased from Qiagen (German). RIPA lysis buffer, bovine serum albumin (BSA), biocinchoninic acid assay (BCA) protein assay kit and loading buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were from Beyotime Biotechnology (Shanghai, China). Cell Counting Kit-8 (CCK8) was bought from Dojindo Molecular Technologies (Kumamoto, Japan). Oligonucleotide primers were from Sangon Biotech (Shanghai, China) and siRNAs were from General Biotech (Anhui, China).

Cell cultures and viability

A549 cells were purchased from ATCC and maintained in RPMI 1640 medium containing 10% FBS in humidified 5% CO₂ at 37°C. CCK-8 assay was utilized to determine cell viability based on the manufacturer’s instructions. Briefly, cells were seeded in 96-well plates and incubated with 10 µL CCK-8 for 2 h at 37 °C. Optical density (OD) at 450 nm was determined using an auto-microplate reader.

RNA interference (RNAi)

Specific small interfering RNAs (siRNA) targeting human HuR mRNA (si-HuR) were as follows: sense (5′-GAGGCAUUACCAGUUUCATT-3′), antisense (5′-UGAAACUGUAUUGCCUUTT-3′). The negative control siRNA (si-NC) sequences were: sense (5′-UUUCUC-CGAACGUACGGUTT-3′), antisense (5′-ACGU-GACACGUUCCGAGAATT-3′). siRNA transfection in A549 cells was performed by lipofectamine-3000 according to the manufacturer’s instructions.

HuR overexpression

To generate HuR expression vector pcDNA3.1-HuR, the HuR coding region was synthesized by Shanghai Gen-ecr biotech Co. Ltd and inserted into a pcDNA3.1 vector. A549 cells were transfected with either pcDNA3.1 vector or pcDNA3.1-HuR with lipofectamine-3000 according to the manufacturer’s instructions.

Scratch assay

After seeding in 24-well plates for 24 h, 1×10⁵ A549 cells were respectively transfected with target si-RNAs (50 pmol) or pcDNA3.1-HuR (500 ng). When 100% confluence was reached and a monolayer was formed, a scratch on the cell monolayer was generated by a sterile 10-µL pipette tip after different treatments. Photographs of the wounds were taken by a phase-contrast microscope (Olympus, Japan) at 0, 24, and 48 h after the scratch.

Transwell assay

After the cells were transfected with si-RNAs or pcDNA3.1-HuR for 24 h, 5×10⁵ A549 cells were resuspended in 200 µL serum-free RPMI-1640 medium in the upper chamber, and 600 µL medium was placed in the bottom chamber. Cell migration was induced by adding a serum to the bottom chambers. The 24-well 8 µm transwell system (Corning, NY, USA) was then incubated for 6 h at 37°C in the presence of 5% CO₂. Cotton swabs were used to gently remove non-migrated cells in the membrane from the upper chamber. Paraformaldehyde (4%) was used to fix the cells that migrated through the pores onto the lower chamber membrane for 30 min. Finally, crystal violet (Beyotime) was used to stain the cells. A phase-contrast
microscope (Olympus) was used to count the cell number in five random fields and expressed as the average number of cells per field.

Quantitative reverse-transcription PCR (qRT-PCR) analysis
After the cells were transfected with si-RNAs or pcDNA3.1-HuR for 24 h, total RNA was extracted from the cells with TRizol. Reverse transcription was conducted with RevertAid H Minus qPCR RT master mix kit based on the manufacturer’s instructions. CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) and the SYBR Green PCR kit were utilized to perform qRT-PCR. Primer sequence information is listed in Table 1. The mRNA level of the house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as the internal control. Experiments were performed in triplicate.

Western-blotting analysis
After 5x10^5 A549 cells were seeded in 6-well plates and transfected with si-RNAs (200 pmol) or pcDNA3.1-HuR (2500 ng) for 24 h, cells were lysed with ice-cold RIPA buffer containing 1% protease inhibitor cocktail. BCA kit was used to determine the total protein concentrations. Total proteins (40 μg) were separated by SDS-PAGE and then transferred onto PVDF membranes (Millipore, MA, USA). After blocking the membranes with 5% milk at room temperature for 1 h, primary antibodies against GAPDH, E-cadherin, Snail and HuR were respectively applied onto the membrane and incubated overnight at 4°C. Tris-buffered saline containing 0.1% Tween-20 (TBST) was used to conduct three 10-min washes, and the membranes were incubated with secondary antibodies conjugated with HRP. An iBright image system (Invitrogen, USA) and ECL reagents were used to detect and analyze the signals. The protein level of β-actin was used as the internal control. Experiments were performed in triplicate. Western-blotting data were quantified using Image J software.

Immunoprecipitation (IP) of ribonucleoprotein complexes
IP of mRNP complexes was performed with a previously described protocol with slight modification (29). Swollen protein-A Sepharose CL-4B bead slurry (20 μL) was used for each IP reaction, which was resuspended in 100 μL NT2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1 mM MgCl2, 0.05% Nonidet P-40) with 5% BSA then incubated with 2 μg anti-HuR or IgG1 isotype control at 4°C for 1 h. For IP, 5x10^5 A549 cells were harvested, pelleted, and resuspended in 360 μL binding buffer (NT2 buffer with 100 U/ml RNasin, 2 mM VRCS, 1 mM DTT, and 15 mM EDTA). Beads were then added to 40 μL of the mRNP-containing lysate. The IP reactions were mixed at room temperature for 2 h to ensure maximal antibody-antigen association. Beads were then washed six times with ice-cold NT2 buffer. Washed beads were resuspended in 100 μL NT2 buffer supplemented with 0.1% SDS and 30 μg of proteinase K and incubated for 30 min at 55°C. The bound RNA was extracted with TRizol. qRT-PCR was utilized to determine the abundance of HuR-associated E-cadherin, Snail, JunB and GADPH mRNAs as described above.

Statistical analysis
Three or more separate experiments were performed for each analysis. All data were expressed as the mean-standard error of mean (SEM). Differences were analyzed with Student’s t-test by GraphPad Prism software. Statistical significance was defined when P<0.05.

Results

HuR knockdown and overexpression
In order to directly investigate the effect of HuR on the migration of A549 cells, HuR knockdown and overexpression cells were constructed by transfection of specific siRNA and overexpression vector, respectively. The cell viability and HuR expressions in A549 cells transfected with si-HuR or HuR overexpression vectors. A, after transfecting A549 cells with 50 pmol si-HuR or si-NC for 24 h, the protein levels of HuR and β-actin were analyzed to determine the relative expression of HuR. B, after transfecting the cells with 500 ng pcDNA3.1-HuR or pcDNA3.1 for 24 h, the protein levels of HuR and β-actin were analyzed to determine the relative expression of HuR. C-D, after transfecting the cells for 24, 48 and 72 h, the cytotoxicity was determined with CCK-8 kit. Statistical significance was represented by * where p<0.05.

Table 1. Sequences of PCR primer pairs.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>GADPH</td>
<td>GGTGGTCTCCTGAGCTGATCCAAACTTCAACA</td>
<td>GAGTTCCGTAGGCCAAATTCGTTG</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>CCACAAAGTCAAGCTCAGATGTTGCAAT</td>
<td>GGAGTTCGGGAAATGTGACG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>GAGAATCTTGCCGCTGAGACG</td>
<td>CTCATGTCAGCGCCTACTC</td>
</tr>
<tr>
<td>Snail</td>
<td>ATCCACAGGTTACCTTCCAG</td>
<td>AGTCCAGATGAGCATTTG</td>
</tr>
<tr>
<td>JunB</td>
<td>ACAACTCTTGAACACGAGCC</td>
<td>CGAGCCCTGACCAGAAAGTA</td>
</tr>
</tbody>
</table>
viability and HuR expression were verified by CCK-8 and Western-blotting (Figure 1). 24 h after transfection with si-HuR, HuR protein level decreased by 85% compared to the cells transfected with RNAi control si-NC (Figure 1A). When the cells were transfected with pcDNA3.1-HuR for 24 h, HuR protein level increased by 40% compared to the cells transfected with control pcDNA3.1 (Figure 1B). After the cells were transfected for 24, 48 and 72 h, the relative cell viability was not significantly different from the control (Figure 1C-D). These results showed that HuR was knocked down or overexpressed successfully in A549 cells without apparently affecting the cell viability.

**HuR affects A549 migration**

HuR knockdown or overexpression A549 cells were subjected to scratch assays in order to determine the effect of HuR on cell migration (Figure 2). For the cells transfected with si-NC control, distinct wound healing was observed after transfection for 24 h and the scratch was almost absent after transfection for 48 h. For the cells transfected with si-HuR for 24 and 48 h, modest wound healing was observed, and the wound healing width was respectively 50% and 40% smaller than the control. For the cells transfected with HuR overexpression vector, the wound healing width was respectively 50% and 35% larger after 24 and 48 h transfection than the control. These results demonstrated that HuR was associated with the migration of A549 cells.

**HuR affects A549 invasion**

Cells transfected with si-HuR or overexpression vector were subjected to transwell assays in order to determine the effect of HuR on A549 invasion (Figure 3). For HuR knockdown cells, the average number of cells per field decreased by 29% compared to the control. For HuR overexpression cells, the number of cells per field increased by 27% compared to the control. These results showed that HuR was associated with the invasion of A549 cells.

**HuR affects E-cadherin and N-cadherin expressions**

In order to investigate the effect of HuR on E-cadherin transcription and translation, qRT-PCR and Western-blotting analyses on E-cadherin and N-cadherin were performed (Figure 4). A, the relative transcription of E-cadherin versus GADPH after A549 cells were transfected with si-HuR or si-NC, and after A549 cells were transfected with pcDNA3.1-HuR or pcDNA3.1 for 24 h. B, the relative transcription of N-cadherin versus GADPH after A549 cells were transfected with si-HuR or si-NC, and after A549 cells were transfected with pcDNA3.1-HuR or pcDNA3.1 for 24 h. C, the relative protein expressions of E-cadherin and N-cadherin versus β-actin were determined after A549 cells were transfected with si-HuR or si-NC for 24 h. D, the relative protein expressions of E-cadherin and N-cadherin versus β-actin were determined after A549 cells were transfected with pcDNA3.1-HuR or pcDNA3.1 for 24 h. Data were means ± SEM from three different experiments. Compared to control, * represents statistical significance where p<0.05.
ing were performed (Figure 4). In the qRT-PCT analysis, the levels of E-cadherin and N-cadherin mRNA were not significantly altered when comparing the cells transfected with si-HuR or overexpression vector (Figure 4A-B) with the control, suggesting that HuR did not influence E-cadherin and N-cadherin transcription. In the Western-blotting analysis, the expression of E-cadherin increased by 1.5-fold while the expression of N-cadherin decreased by 50% comparing the cells transfected with si-HuR to the si-NC control (Figure 4C), and the expression of E-cadherin decreased by 51% while the expression of N-cadherin increased by 1.8-fold in HuR overexpression cells compared to the control (Figure 4D), demonstrating that HuR affected E-cadherin and N-cadherin expressions in A549 cells.

**HuR affects Snail expression and interacts with E-cadherin and Snail mRNA**

In order to investigate the effect of HuR on Snail expression and to determine the interactions of HuR with E-cadherin, N-cadherin and Snail mRNA, qRT-PCR, Western-blotting and IP analyses were conducted (Figure 5). In the qRT-PCT analysis, Snail mRNA level in the cells transfected with si-HuR (Figure 5A) or HuR overexpression vector (Figure 5B) was not significantly different from the control, suggesting that HuR did not influence Snail transcription. In the Western-blotting analysis, the expression of Snail was reduced by 38% when comparing the cells transfected with si-HuR to si-NC (Figure 5C) and increased by 83% when comparing HuR overexpression cells with the control (Figure 5D), demonstrating that HuR affected Snail expression in A549 cells. In IP analysis, the mRNA fold enrichment for positive control JunB, E-cadherin and Snail mRNA in HuR containing ribonuclear protein complex was approximately 200, 95 and 15, respectively, while the enrichment for negative control GAPDH was 0.008 (Figure 5E), and N-cadherin mRNA was not detected in the HuR mRNP complex. These results supported that HuR could directly interact with E-cadherin and Snail mRNA in A549 cells.

**Discussion**

In the present study, we have validated that HuR knockdown or overexpression reduced or increased HuR expression, respectively, and did not affect the viability of A549 cells. In the HuR knockdown cells, the migration of A549 cells was apparently inhibited and the number of invasive cells was significantly decreased, whereas, in the HuR overexpression cells, opposite effects were observed, substantiating the effect of HuR on A549 migration and invasion. E-cadherin expression was upregulated and N-cadherin expression was downregulated when HuR was knocked down, while E-cadherin expression was downregulated and N-cadherin expression was upregulated when HuR was overexpressed, suggesting that HuR affected A549 migration by modulating the expressions of EMT markers. In addition, we have also demonstrated that HuR directly interacted with the E-cadherin repressor Snail, and influenced its expression in A549 cells, indicating that HuR stabilizes Snail expression to downregulate E-cadherin level, which in turn facilitates the migration and invasion of lung cancer cells.

HuR is a multifaceted post-transcriptional regulator involved in a wide range of both physiological and pathological processes. Under normal physiological conditions, HuR is typically located inside the nucleus to fulfill its regulatory functions. Genetic deletion of HuR gene was found to inhibit growth and skeletal development and suppress immune and hematopoietic progenitor cell survival in mice (30,31). In addition, HuR was also demonstrated to influence adipogenesis, myogenesis, spermatogenesis as well as atherosclerosis (32-35). These normal physiological processes are highly dependent on cell proliferation and migration, which to a certain degree support our finding that HuR is involved in A549 migration.

In the context of pathological and stressful conditions (oxidations, inflammations, radiations, viral infections, chemical and hormone disturbances, etc.) or with the cues from cancerous proliferative signals, HuR is relocated from the nucleus to the cytoplasm to stabilize target mRNAs and affect the processing of corresponding transcripts. Accordingly, HuR is reported to be highly associated with the pathogenesis of the inflammatory disorder, multiple sclerosis, diabetic nephropathy, and especially malignancies (36-39). Due to the critical role in cell proliferation, abnormal overexpression of HuR was identified.
in a number of solid tumorous tissues, including lung, liver, breast and colon (40-43). However, direct evidence in A549 cells was not previously documented. In the present study, by knocking-down or overexpressing HuR directly, our results showed that HuR could promote the migration and invasion of A549 cells, highlighting the important role of HuR in lung cancer progression and metastasis.

The progression of cancer and the effectiveness of metastasis are tightly associated with EMT, in which E-cadherin downregulation and N-cadherin upregulation play an essential role due to their primary function in maintaining the epithelial cell-to-cell interactions. In gastric cancer cell lines, interleukin (IL)-17a and methyltransferase-like 3 were shown to promote EMT and repress E-cadherin expression via HuR pathway (19,44). In human colorectal adenocarcinoma Caco-2 cells, HuR overexpression was shown to prevent CUG-binding protein 1-induced E-cadherin repression, while HuR depletion was found to increase the miR-675 level, which in turn suppressed the expression of E-cadherin (17,18). In esophageal squamous cancer cells, HuR knockdown was reported to inhibit cell proliferation, disturb cell cycle, increase E-cadherin expression and suppress metastasis, possibly via IL-18, a HuR-binding target (16). In agreement, findings from the present study showed that HuR facilitated the migration and invasion of A549 cells by decreasing E-cadherin expression and increasing N-cadherin expression. Interestingly, unlike esophageal cancer cells, the viability of A549 cells was not significantly altered when HuR was knocked down or overexpressed, possibly due to the heterogeneity and varying competency of different cancer cell lines, indicating that it would be informative to independently explore the effect of HuR in various types of cancer. Although the relationship between HuR and E-cadherin was elaborated in a number of cancerous cell lines, to our knowledge, this study provides the first preliminary evidence in lung cancer A549 cells, and limited studies were performed in normal bronchial epithelial BEAS-2B cells, aiming to evaluate the effect of cigarette smoke extract on chronic obstructive pulmonary disease (14,15). Thus, our results would provide an additional reference for studying the pathogenesis of lung cancer.

HuR is a post-transcriptional RBP that typically binds and stabilizes target transcripts, resulting in an elevated translation. Accordingly, the observed downregulation of E-cadherin expression might not be the direct consequence of HuR overexpression, but likely via an E-cadherin repressor upregulated by HuR. In human gastric cancer AGS cells, an increased level of HuR was associated with promoted Snail translation, leading to reduced E-cadherin expression and enhanced EMT, while these effects were reversed in HuR knockdown cells (19). Similarly, HuR was shown to directly interact with the 3'-UTR of the Snail mRNA, stabilizing Snail expression and promoting EMT in pancreatic cancer MIA PaCa-2 cells (20). In our study, IP analysis demonstrated that HuR could bind to Snail mRNA in lung cancer A549 cells, and the stabilization and upregulation of Snail induced by HuR were accompanied by a reduced E-cadherin level, suggesting that the effect of HuR on E-cadherin expression and A549 migration might be realized via Snail. In fact, a recent impactful report showed that the association of HuR with SNAI1 mRNA was inhibited by uridine diphosphate (UDP)-glucose, and phosphorylated UDP-glucose 6-dehydrogenase, a UDP-glucose metabolizing enzyme, strengthened the binding of HuR to Snail in lung cancer cells, thereby promoting lung cancer metastasis (23). Interactions between HuR and Snail mRNA were also observed in human small airway epithelium, well-differentiated and poorly-differentiated breast cancer cells (15,45,46). In addition, HuR and Snail were shown to play a critical role in Scribble-mediated cancer drug resistance (47). These findings together with our observation would strongly support the current ongoing drug development and delivery strategy explorations targeting HuR (28).

In conclusion, this study provided direct evidence that HuR influenced the migration and invasion of lung cancer A549 cells and regulated the expressions of E-cadherin, N-cadherin and Snail. Future investigations should focus on identifying factors affecting HuR expression in various cancers, and developing HuR-related anti-cancer strategies.

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Conflict of interest
The authors declare that there is no conflict of interest.

Author’s contribution
Shufang Shan and Qixue Bao: investigation, data analyzing, and manuscript writing. Guochen Ma and Yuqin Yao: data analysis, technical support and manuscript reviewing. Jingyuan Xiong and Jia You: conceptualization, funding acquisition, and manuscript writing, editing and reviewing.

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