The effects of modified RNA-binding proteins HuR on the biological behavior of the bladder cancer T24 cell line

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Background: In tumors, the role of human antigen R (*HuR*) includes regulating tumor cell proliferation, differentiation, apoptosis, angiogenesis, and lymphangiogenesis. Previous studies have revealed that the expression of *HuR* can be detected in bladder cancer, and is related to the biological behavior of malignancy.

Methods: T24 cells were transfected by *HuR* overexpression and *HuR* knockdown vectors, and divided into the control group, the overexpression-*HuR* group, and the cas9-*HuR* group. Cell viability was detected after 48 h by methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, apoptosis was detected by Annexin V-allophycocyanin (APC)/7-aminoactinomycin D (7-AAD) double staining, cell migration was detected by Transwell assays, and the expression levels of *HuR*, *cyclin D1*, and apoptosis-related factors [i.e., *B-cell lymphoma 2* (*Bcl-2*)] were detected by fluorescence quantitative polymerase chain reaction (PCR) and Western blot.

Results: Compared to the control group, cell viability after 48 h increased significantly in the overexpression-*HuR* group, and decreased significantly in the cas9-*HuR* group (P<0.05). The number of migrating cells increased significantly in the overexpression-*HuR* group, and decreased significantly in the cas9-*HuR* group (P<0.05). The apoptosis rate was significantly decreased in the overexpression-*HuR* group, and significantly increased in the cas9-*HuR* group (P<0.05). The messenger ribonucleic acid and protein expression levels of *HuR*, *cyclin D1*, and *Bcl-2* were significantly increased in the overexpression-*HuR* group, and significantly decreased in the cas9-*HuR* group (P<0.05).

Conclusion: *HuR* promotes the proliferation and migration of T24 cells, and inhibits cell apoptosis. The mechanism may be related to the expression of *cyclin D* and the apoptosis-related protein, Bcl-2.

Keywords: Bladder cancer; human antigen R (*HuR*); T24 cell line

Introduction

Bladder cancer is one of the most common types of cancers of the urinary system (1). It is estimated that >300,000 patients are diagnosed with bladder cancer every year, and >100,000 die from bladder cancer worldwide (1,2). At present, the main therapy for bladder cancer is a comprehensive treatment mode comprising a combination of surgery, radiotherapy, systemic chemotherapy, and immunity therapy. However, the majority of patients relapse after treatment, and often also suffer from a deterioration in condition (2), and the molecular mechanisms of bladder cancer recurrence and metastasis remain unclear. Although many signaling pathways, such as PI3K/AKT/mTOR, Wnt/β-catenin, and NF-κB star signaling pathways, are involved in the molecular regulation of bladder cancer recurrence and metastasis, due to the complex interaction between different signaling pathways, there is no clear evidence to fully reveal molecular mechanisms of recurrence and metastasis of bladder cancer (3). Thus, further research needs to be conducted on the genetic mechanisms of bladder cancer for therapeutic method development.

Human antigen R (HuR) is a ribonucleic acid (RNA)-binding protein in the embryonic lethal abnormal vision (ELAV) family. In tumors, the role of *HuR* includes regulating tumor cell proliferation, differentiation, apoptosis, angiogenesis, and lymphangiogenesis (4-7). Previous studies have revealed that the expression of *HuR* can be detected in ovarian cancer, gastric cancer, breast cancer, cervical cancers, and other tumor tissues, and overexpression *HuR* in cancer cells has been associated with poor prognosis and resistance to radiotherapy and chemotherapy (4-8). In bladder cancer, high cytoplasmic HuR expression was noticed in cancer tissues, and appeared positively associated with Pathologic TNM stage and grade (9). However, the rare research was published for molecular mechanism of HuR in bladder cancer recurrence and metastasis. Hence, the role and molecular mechanism of HuR in bladder cancer still needs to be further studied and revealed.

Cyclin D1 acts as a regulator of cyclin-dependent kinase (CDKs), and its main function is to promote cell proliferation (10). Among the apoptosis-related proteins, the B-cell lymphoma 2 (BCL-2) family of proteins is the most studied, and is also the most important class of anti-apoptotic protein (11,12). HuR can bind to the adenylate-uridylate-rich element (ARE) fragment of the messenger RNA (mRNA) 3'-untranslated region (3'UTR) of various

genes (12). The mRNA encoding the cyclin D1 has been experimentally shown to contain an ARE fragment, and HuR may enhance the stability of the factor mRNA by binding to ARE, and thereby upregulating the expression of the above-mentioned factor protein and exerting the corresponding biological effects. Thus, the present study employed human bladder cancer T24 cells to explore the effects of *HuR* on the behavior of bladder cancer cells, and examine the relationship between the expression of *HuR* and *cyclin D1* and *Bcl-2*. We present the following article in accordance with the MDAR reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-123/rc).

Methods

Experimental materials

The T24 human bladder transitional cell carcinoma cell line was purchased from Procell Co., Ltd., and mycoplasma testing was conducted for all cells. We also purchased the following: Roswell Park Memorial Institute (RPMI)-1640 cell culture medium, and trypsin, fetal bovine serum, 1% streptomycin (Gibco; Thermo Fisher Scientific, Inc.); pU6gRNACas9EGFP vector and pIRES2-ZsGreen vector (Addgene, Inc); Lipofectamine 2000™ transfection reagent, TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.); reverse transcription (RT)–polymerase chain reaction (PCR) kits, T4 deoxyribonucleic acid (DNA) ligase, restriction endonuclease *Xho* I, and *Eco*R I (Takara Biotechnology, Co., Ltd.); DNA gel recovery kits (Sangon Biotech, Co., Ltd.); Transwell Chamber (Corning, Incorporated); Radio immunoprecipitation assay (RIPA) lysate, bicinchoninic acid (BCA) protein concentration kits, and methylthiazolyldiphenyl-tetrazolium bromide (MTT) test kits (Beyotime, Institute of Biotechnology); apoptosis detection kits (Nanjing KeyGEN BioTECH, Co., Ltd.); HuR antibody, cyclin D1 antibody, and BCL-2 antibody (Abcam); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (ProteinTech Group, Inc.).

Cell culture

The T24 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% streptomycin, and grown at 37 ℃ and 5% carbon dioxide (CO_2). The cells were routinely subcultured when they reached ~80% confluence.

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Table 1 Primer sequences of *human antigen R* (*HuR*), *cyclin D1*, *B-cell lymphoma 2* (*Bcl-2*), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

Name	Primer	Sequence	Size
GAPDH	Forward	5'-TCAAGAAGGTGGTGAAGCAGG-3'	115 bp
	Reverse	5'-TCAAAGGTGGAGGAGTGGGT-3'	
HuR	Forward	5'-TCATCTACAACCTGGGGCAG-3'	162 bp
	Reverse	5'-CCATCGCGGCTTCTTCATAG-3'	
Cyclin D1	Forward	5'-CGGACTACAGGGGAGTTTTG-3'	273 bp
	Reverse	5'-AGGAGGTTGGCATCGGGGT-3'	
Bcl-2	Forward	5'-GCCTTCTTTGAGTTCGGTGG-3'	192 bp
	Reverse	5'-GAAATCAAACAGAGGCCGCA-3'	

Construction of the HuR overexpression vector

The total RNA of the T24 cells was extracted according to the TRIzol kit's instructions, complementary DNA (cDNA) was obtained by RT-PCR, and PCR was performed using the following cDNA as a template: Premix Taq 12.5 μL, 1 μL of upstream and downstream primers, 2 μL of template, and ddH$_2$O in 25 μL of reaction system. The thermocycling conditions were 94 ℃ for 5 min, 30 cycles of 94 ℃ for 30 sec, 61 ℃ for 30 sec and 72 ℃ for 2 min, and then a 72 ℃ extension for 10 min. After electrophoresis, the gel was recovered to obtain the *HuR* gene fragment. The recovered *HuR* fragment and vector pIRES2-ZsGreen were digested with the restriction endonucleases *Xho* I and *Eco*R I, and the digested product was constructed with T4 DNA ligase to construct a *HuR* expression vector. The constructed *HuR* overexpression vector was then identified by DNA sequencing.

Construction of the HuR knockdown vector

The guide RNA (gRNA) of the *HuR* gene (sequence: 5'-AGAGCGATCAACACGCTGAA-3'; synthesized by Shanghai GenePharma Co., Ltd) was designed using the clustered regularly interspaced short palindromic repeats (CRISPR) online design tool (http://crispr.mit.edu/). After annealing to a double strand, the clone was ligated into the pB6gRNACas9EGFP vector digested with *Bbs* I. After transformation into competent escherichia coli (*E. coli*) DH5α, the monoclonal extraction plasmid was picked and sequenced to verify that the correctly cloned plasmid was obtained.

Quantitative (q)-PCR to detect the mRNA expression of HuR, cyclin D1, and Bcl-2

After 48 h of transfection, the control, *HuR* overexpression, and *HuR* knockdown groups were collected, and the total RNA was extracted according to the TRIzol kit's instructions. The cDNA was obtained by RT-PCR, and the cDNA was used as a template for quantitative PCR. GAPDH was selected as the internal reference, and the relative expression of *HuR*, *cyclin D1*, and *Bcl-2* mRNA was determined by the $2^{-\Delta\Delta Cq}$ relative quantitative method. The primer sequences are presented in *Table 1*. Three independent experiments were repeated.

Western blot of analysis of HuR, cyclin D1, and Bcl-2

The cells of the control, *HuR* overexpression, and *HuR* knockdown groups were collected after 48 h. The cell lysate was lysed at 4 ℃, and centrifuged at 12,000 r/min for 30 min, and the supernatant was aspirated to obtain total protein. According to the quantitative results of the BCA assay, 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis was conducted for 2.5 h. After the proteins were transferred to the membrane, the negative control membrane was washed in tris-buffered saline (TBS). The membranes were then blocked with 5% skim milk for 1 h at room temperature. Antibodies against HuR, cyclin D1, and Bcl-2 were incubated at 4 ℃ overnight, and the membranes were then washed for 10 min repeatedly for 3 times in TBS with Tween (TBST). The secondary antibodies were incubated for 1 h at room temperature, and then washed with TBST for 10 min 3 times. Next, the

membranes were visualized by exposure development. A gray value analysis was performed using BIO-RAD Image Lab software.

MTT assays for cell proliferation activity

The MTT assays were performed 48 h after cell transfection. The control, *HuR* overexpression, and *HuR* knockdown groups were seeded in a 96-well plate. A total of 10 µL of MTT was added to each well. After incubating at 37 ℃ for 4 h, the medium was aspirated, and 150 µL of dimethyl sulfoxide was then added, and shaken on a shaker for 10 min. The absorbance value of the optical density of each well at a wavelength of 568 nm was measured with a microplate reader. Each experiment group had three replicate, and three independent experiments were repeated.

Apoptosis detection via Annexin V-APC/7-AAD double staining

The cells of the control, *HuR* overexpression, and *HuR* knockdown groups were collected at 48 h after transfection and then washed 2–3 times with pre-cooled phosphate buffered solution (PBS). The cells were adjusted to a cell concentration of $5×10^5$/mL, and resuspended via the addition of 100 µL binding buffer. Next, 5 µL of Annexin V-APC and 5 µL of 7-AAD solution were added and mixed for 10 min at room temperature. Finally, 400 µL of 1X binding buffer was added. The samples were analyzed by flow cytometry. Three independent experiments were repeated.

Transwell assays for cell migration

The cells of the control, *HuR* overexpression, and *HuR* knockdown groups were collected 48 h after cell transfection. The cell concentration was diluted to $2× 10^5$/mL with serum-free medium. Next, 800 µL of 10% fetal bovine serum containing medium was added to a 24-well plate and placed in a Transwell chamber. Next, 200 µL of each cell suspension was inserted into the Transwell chamber, and cultured at 37 ℃ in a 5% CO_2 incubator for 48 h. The Transwell insert was then removed, the chamber was carefully washed with PBS, and the cells were fixed with 70% ice ethanol solution for 1 h. The cells were stained with 0.5% crystal violet staining solution, left at room temperature for 20 min, washed with PBS, and the

unmigrated cells on the upper chamber were then wiped clean with a clean cotton ball. Pictures were taken under a microscope, and the number of cells was counted. Three independent experiments were repeated.

Statistical analysis

All the experiments were repeated at least 3 times. The statistical analysis was performed using SPSS 23.0 statistical software (SPSS, Inc.) The data are expressed as the mean ± standard deviation, and an analysis of variance and a post-hoc test for multiple comparisons were used for comparisons between groups. A P value<0.05 indicated a statistically significant difference.

Results

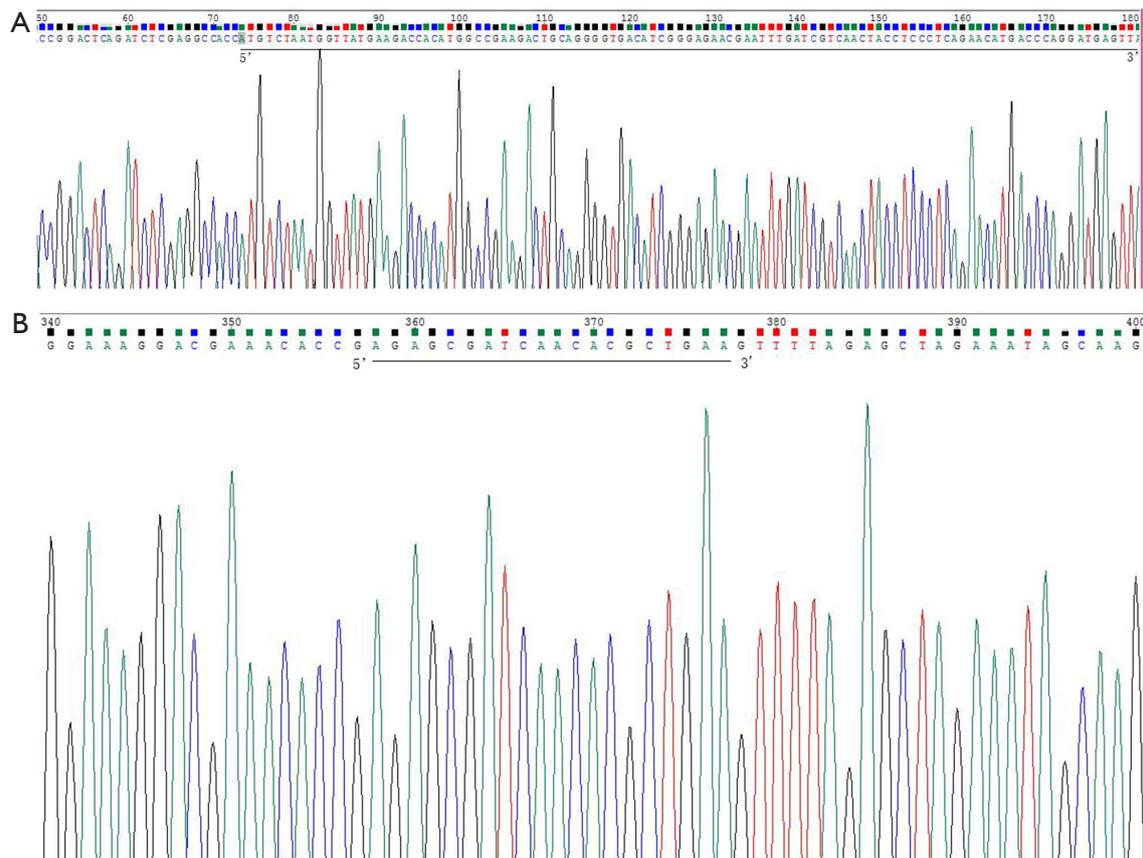### Construction of the HuR overexpression vector and knockdown vectors

As *Figure 1A,1B* show, the constructed *HuR* overexpression and knockdown vectors were sequenced and analyzed, which indicated that the inserted sequence and site were correct, and the overexpression of the target gene *HuR* and the knockdown vector plasmid had been successfully constructed.

Silencing and overexpression of HuR in response to HuR transfection and HuR knockdown by CRISPR-associated proteins 9 (cas9)
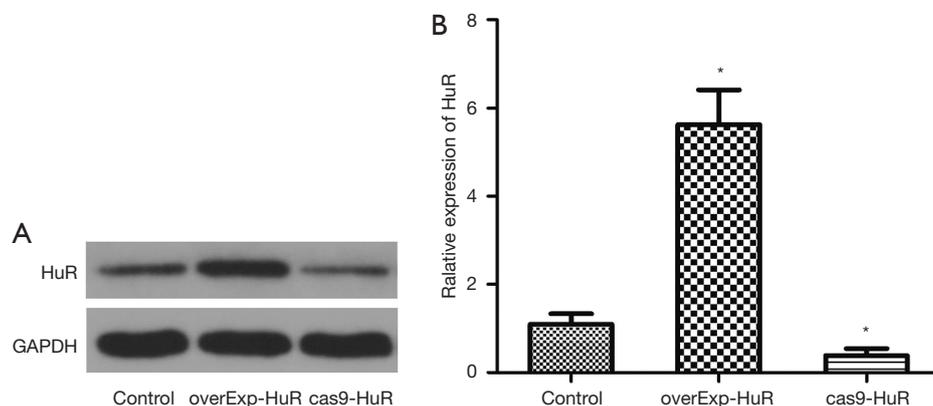
The protein expression levels of HuR were detected by western blotting, and are set out in *Figure 2A*. Compared to the control group, the expression level of HuR was significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group (P<0.01). The relative expression levels of mRNA of *HuR* were detected by PCR (see *Figure 2B*). Compared to the control group, the expression level of *HuR* was significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group (P<0.01).

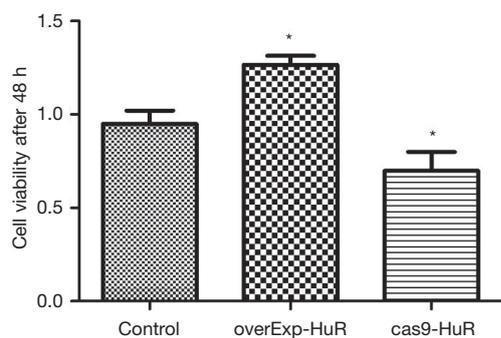### HuR promotes the proliferation of T24 cells

Compared to the control group, after 48 h, the cell viability was significantly increased in the *HuR* overexpression cells, and significantly decreased in the *HuR* knockdown group

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Figure 1 Construction of the *human antigen R* (*HuR*) overexpression and knockdown vectors. The constructed *HuR* overexpression (A) and knockdown (B) vectors were sequenced and analyzed.

Figure 2 The silencing and overexpression of *human antigen R* (*HuR*) in response to *HuR* transfection and *HuR* knockdown by cas9. (A) The protein expression levels of HuR were detected by western blotting; compared to the control group, the expression level of HuR was significantly increased in the *HuR* overexpression group and significantly decreased in the *HuR* knockdown group (F=55.890, P<0.01). (B) The relative expression levels of mRNA of *HuR* were detected by fluorescence quantitative polymerase chain reaction (PCR). Compared to the control group, the expression level of *HuR* was significantly increased in the *HuR* overexpression group and significantly decreased in the *HuR* knockdown group (F=124.230, P<0.01). *, P<0.05.

Figure 3 *Human antigen R* (*HuR*) promotes the proliferation of T24 cells. Compared to the control group, after 48 h, cell viability was significantly increased in the *HuR* overexpression group and significantly decreased in the *HuR* knockdown group (F=1929.061, P<0.01). *, P<0.05.
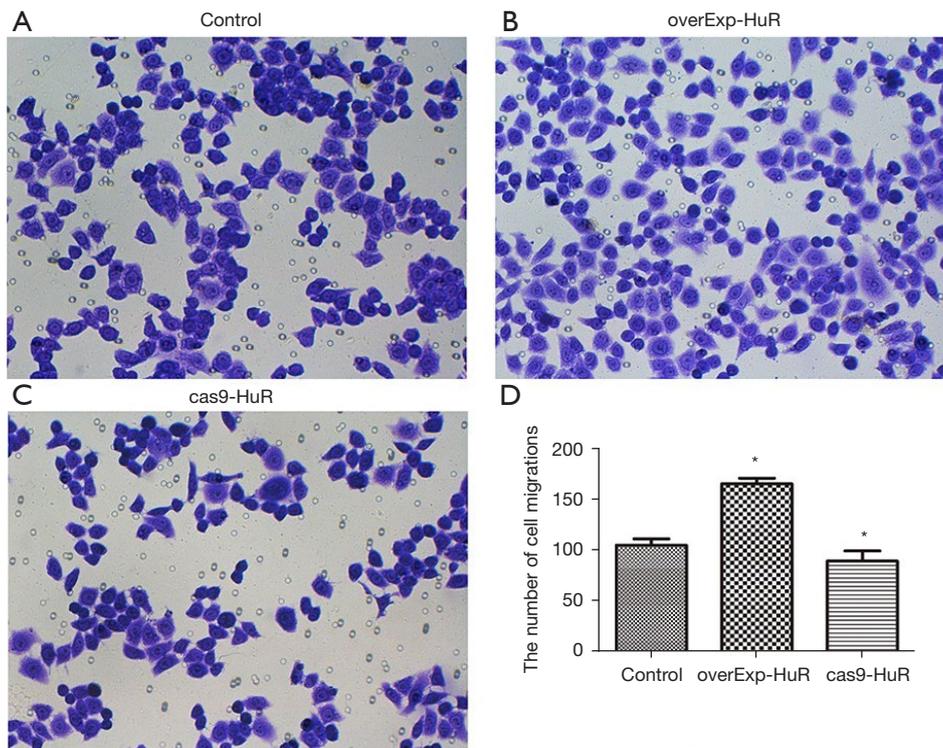
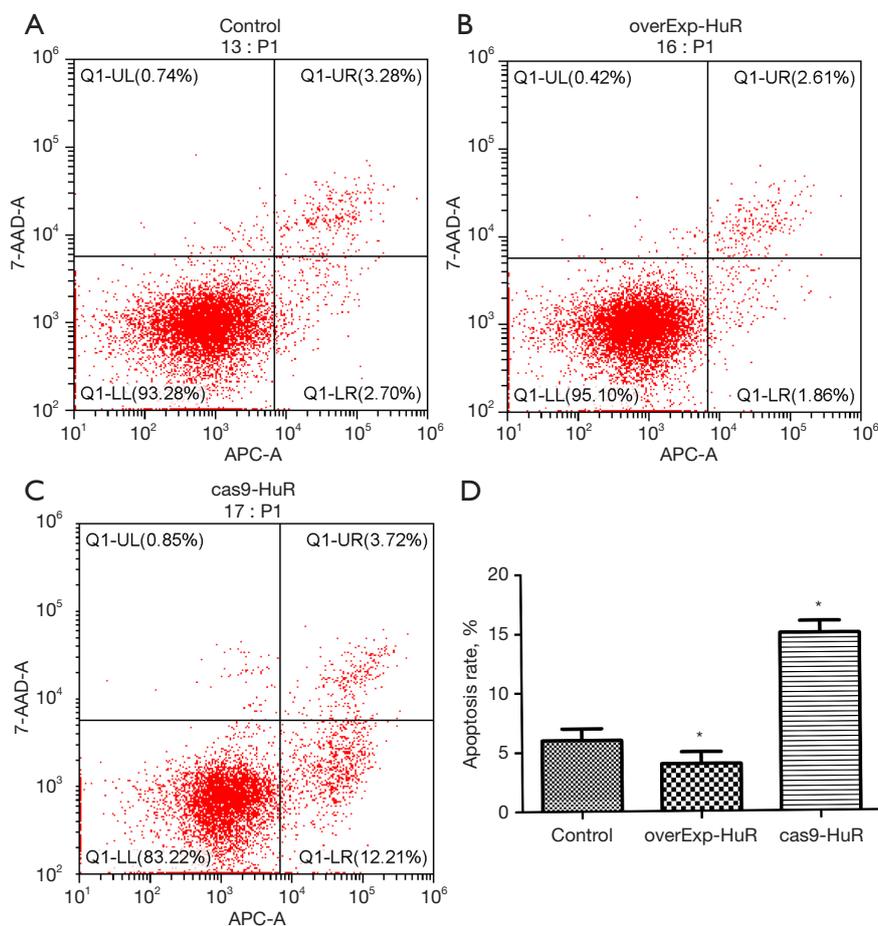(P<0.01; see *Figure 3*).

HuR promotes the migration of T24 cells

Compared to the control group, the number of migrated cells was significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group (P<0.01; see *Figure 4*).

HuR inhibits T24 cell apoptosis

Compared to the control group, the apoptotic rate was significantly decreased in the *HuR* overexpression group, and significantly increased in the knockdown group (P<0.01; see *Figure 5*).

Figure 4 *Human antigen R* (*HuR*) promotes the migration of T24 cells. Compared to the control group, the number of migrated cells of *HuR* overexpression group was significantly increased and significantly decreased in the *HuR* knockdown group (F=145.982, P<0.01, all the photos magnified at ×400; all the cells were stained by crystal violet staining). *, P<0.05. (A) Control group; (B) *HuR* overexpression group; (C) *HuR* knockdown group; (D) the number of cell migrations of each group.

Figure 5 *Human antigen R* (*HuR*) inhibits T24 cell apoptosis. Compared to the control group, the apoptotic rate was significantly decreased in the *HuR* overexpression group and significantly increased in the knockdown group (F=665.452, P<0.01). *, P<0.05. (A) Control group; (B) *HuR* overexpression group; (C) *HuR* knockdown group; (D) each group apoptosis rate of T24 cell.
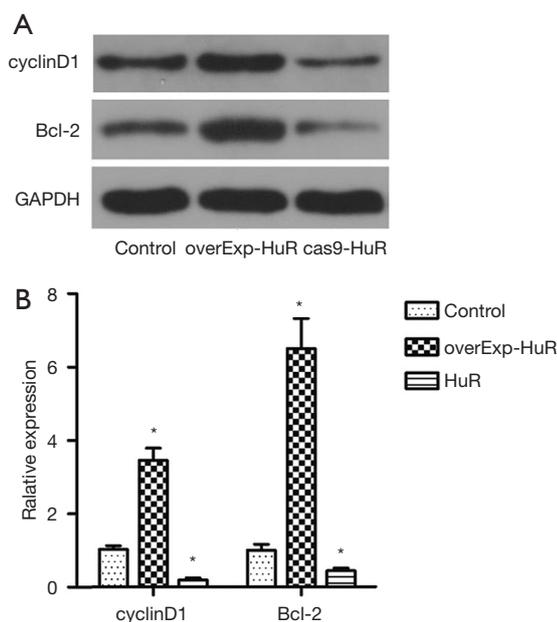
Effect of HuR on the expression of cyclin D1 and Bcl-2

The expression levels of the proteins of cyclin D1 and Bcl-2 were detected by western blotting (see *Figure 6A*). Compared to the control group, the expression levels of cyclin D1 (P<0.01) and Bcl-2 (P<0.01) were significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group.

The relative expression levels of mRNA of *cyclin D1* and *Bcl-2* were detected by PCR (see *Figure 6B*). Compared to the control group, the expression levels of cyclin D1 (P<0.01) and Bcl-2 (P<0.01) were significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group.

Discussion

The progression of tumors is the result of a series of interactions between multiple genes in which tumor gene expression and function regulation have altered levels that can be regulated at the pre-transcriptional, transcriptional, and post-transcriptional levels (13-15). At the post-transcriptional level, there is a class of proteins that regulate the metabolic processes of RNA and bind to RNA, which affects the metabolism of RNA. These proteins are called RNA-binding proteins (RBPs), of which HuR is a widely studied type (16-18). HuR is a member of the ELAV family of RNA-binding proteins, located at 19p13.2, which encodes a variety of proto-oncoproteins (16-18). HuR

Figure 6 The effects of *human antigen R* (*HuR*) on the expression of *cyclin D1* and *Bcl-2*. (A) The expression levels of protein of cyclin D1 and Bcl-2 were detected by western blotting. Compared to the control group, the expression levels of cyclin D1 (F=49.102.762, P<0.01) and Bcl-2 (F=265.736, P<0.01) were significantly increased in the *HuR* overexpression group and decreased in the *HuR* knockdown group. (B) The relative expression levels of mRNA of *cyclin D1* and *Bcl-2* were detected by fluorescence quantitative polymerase chain reaction (PCR). Compared to the control group, the expression levels of *cyclin D1* (F=225.681, P<0.01) and *Bcl-2* (F=202.762, P<0.01) were significantly increased in the *HuR* overexpression group and decreased in the *HuR* knockdown group. *, P<0.05.

binds to and stabilizes a partial fragment in the 3'UTR, which is widely expressed in the cytoplasm and nucleus of mammalian cells, and is mainly expressed in the nucleus in normal tissue cells. In tumor cells, there is a positive increase in HuR expression, especially in the cytosol (19,20). Young *et al.* reported that HuR was significantly more highly expressed in colon tumor tissues than normal colon tissues, and the increased expression of HuR promoted the development of colon cancer (21). Heinonen *et al.* demonstrated that in breast cancer patients with the non-breast cancer susceptibility gene 1/2 mutation, the positive rate of cytoplasmic HuR was 39.4%, and the high expression of HuR was closely associated with the number of lymph node metastases (22,23). The experimental results

also suggested that HuR may be involved in lymph node metastasis (22,23). A recent study revealed that HuR was highly expressed in various tumor tissues, such as breast, gastric, esophageal, and ovarian cancer tissues (24). In the present study, the proliferation rate and migratory ability of *HuR* overexpression cells were significantly increased, and the apoptotic rate was decreased. The proliferation rate and migratory ability of *HuR* knockdown cells were significantly decreased, and the apoptotic rate was significantly increased. Thus, *HuR* was shown to promote the proliferation and migration and decrease the apoptosis of human bladder cancer T24 cells.

The *cyclin D1* gene is a proto-oncogene that plays a very important regulatory role in the cell cycle of eukaryotic cells (25,26). Cyclin D1 binds and activates cyclin dependent kinase 4 (CDK4), which is a characteristic of the G1 phase. The G1 cycle inhibitory protein [retinoblastoma protein (Rb)] is phosphorylated, and the phosphorylated Rb protein is then cleaved from its bound early 2 factor (E2F) transcription factor; in turn, the E2F transcription factor initiates the transcription of the cell-cycle gene and forms a protein complex with a cell cycle-dependent kinase, and thus, the cells pass through the cell cycle G1/S control point and enter the S phase (25,26). The expression of *cyclin D1* has been shown to be significantly increased in many tumor tissues, such as breast cancer, nasopharyngeal carcinoma, lung cancer, and liver cancer tissues (27-30).

In the present study, the expression of *cyclin D1* was significantly increased in the *HuR* overexpression group, and significantly decreased in the *HuR* knockdown group, which suggests that the mechanism of HuR on the biological behavior of T24 cells may be related to the expression of *cyclin D1* and the cell cycle. The *Bcl-2* gene is also a proto-oncogene that alters the permeability of the mitochondrial outer membrane and inhibits the release of cytochrome C into the cytoplasm, thereby preventing the activation of the caspase cascade and the inhibition of apoptosis. The level of Bcl-2 protein expression regulates apoptosis. When Bcl-2 expression is high, apoptosis is inhibited. Conversely, when Bcl-2 expression is low, apoptosis is promoted. The present study revealed that compared to the control group, the expression of *Bcl-2* was significantly increased in the *HuR* overexpression group, and the expression of *Bcl-2* was significantly decreased in the *HuR* knockdown group, which suggests that HuR inhibits the apoptosis of human bladder cancer T24 cells, which is consistent with the Conclusions that HuR inhibits apoptosis. It is indicated that HuR plays a key role in the occurrence and development of

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bladder cancer and may serve as a potential target for the development of new anti-bladder cancer drugs.

Conclusions

In conclusion, the RNA-binding protein HuR promotes the proliferation and migration of bladder cancer T24 cells, and inhibits apoptosis, and the mechanism may be related to its effect on the cell cycle and apoptosis. The expression of *HuR* is related to cyclin D1 and the apoptosis-related protein Bcl-2. Thus, the *HuR* gene may be a novel target gene for targeted therapy in bladder cancer patients. However, this study had some limitations. First, only one bladder cancer cell line was examined in this study. Second, experiments of both animal and human tissue samples were not conducted. Third, due to a lack of finance, only 1 apoptosis-related protein was selected for this experiment. Thus, further research, especially *in-vivo* studies, need to be conducted in the future.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-22-123/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-22-123/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-123/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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