

Effect of modified RNA-binding proteins HuR on biological behavior of bladder cancer T24 cell line

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Background: In tumors, the role of human antigen R (HuR) involves regulating tumor cell proliferation, differentiation, apoptosis, angiogenesis, and lymphangiogenesis. Previous studies have revealed the expression of HuR can be detected in bladder cancer and related to biological behavior of malignancy. **Methods:** T24 cells were transfected by HuR overexpression vector and HuR knockdown vector. The cells were divided into control group, overExp-HuR group and cas9-HuR group. The cell viability after 48 h was detected by MTT, the apoptosis was detected by Annexin V-APC/7-AAD double staining, the cell migration was detected by Transwell assay, and the expression of HuR, cyclinD1 and apoptosis-related factors (Bcl-2) were detected by fluorescence quantitative PCR and Western blot. **Results:** Compared with the control group, the cell viability after 48 h in the overExp-HuR group increased significantly, and decreased in the cas9-HuR group ($P < 0.05$). And the number of migrating cells increased in the overExp-HuR group, and decreased in the cas9-HuR group ($P < 0.05$). The apoptosis rate of the overExp-HuR group decreased significantly, and increased significantly in the cas9-HuR group ($P < 0.05$). The mRNA and protein expression of HuR, cyclinD1 and Bcl-2 of the overExp-HuR group increased, and decreased significantly in cas9-HuR group ($P < 0.05$). **Conclusion:** HuR promote the proliferation and migration of T24 cells, and inhibit cell apoptosis. And the mechanism may be related to the expression of cyclin D and apoptosis-related proteins Bcl2.

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Abstract

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

Methods: T24 cells were transfected by HuR overexpression vector and HuR knockdown vector. The cells were divided into control group, overExp-HuR group and cas9-HuR group. The cell viability after 48 h was detected by MTT, the apoptosis was detected by Annexin V-APC/7-AAD double staining, the cell migration was detected by Transwell assay, and the expression of HuR, cyclinD1 and apoptosis-related factors (Bcl-2) were detected by fluorescence quantitative PCR and Western blot.

Results: Compared with the control group, the cell viability after 48 h in the overExp-HuR group increased significantly, and decreased in the cas9-HuR group ($P < 0.05$). And the number of migrating cells increased in the overExp-HuR group, and decreased in the cas9-HuR group ($P < 0.05$). The apoptosis rate of the overExp-HuR group decreased significantly, and increased significantly in the cas9-HuR group ($P < 0.05$). The mRNA and protein expression of HuR, cyclinD1 and Bcl-2 of the overExp-HuR group increased, and decreased significantly in cas9-HuR group ($P < 0.05$).

Conclusion: HuR promote the proliferation and migration of T24 cells, and inhibit cell apoptosis. And the mechanism may be related to the expression of cyclin D and apoptosis-related proteins Bcl2.

Introduction

Bladder cancer is one of the most common types of cancers of the urinary system (1.Torre L A et al., 2015) . In total, >300,000 patients are diagnosed with bladder cancer every year, and >100,000 die from bladder cancer worldwide (1.Torre L A et al., 2015; 2. Burger M et al., 2013). At present, the main therapy of bladder cancer is comprehensive treatment mode combined with surgical, radiotherapy, systemic chemotherapy and immunity therapy. However, the majority of patients relapse after treatment, which is often accompanied by deterioration of the condition (2. Burger M et al., 2013). Therefore, further research is required on the genetic mechanisms of bladder cancer for therapeutic method development. Human antigen R (HuR) is an RNA-binding protein in the embryonic lethal abnormal vision (ELAV) family. In tumors, the role of HuR involves regulating tumor cell proliferation, differentiation, apoptosis, angiogenesis, and lymphangiogenesis (3. Dong R et al., 2007;4. Kang M J et al., 2008; 5. Huang Y H et al., 2016; 6. Cho N H et al., 2006). Previous studies have revealed that the expression of HuR can be detected in ovarian cancer, gastric cancer, breast and cervical cancers as well as in other tumor tissues (3. Dong R et al., 2007;4. Kang M J et al., 2008; 5. Huang Y H et al., 2016; 6. Cho N H et al., 2006). Cyclin D1 acts as a regulator of cyclin-dependent kinase (CDKs), and the main function of cyclinD1 is to promote cell proliferation (7. Qie S et al., 2016). Among the apoptosis-related proteins, the BCL-2 family of proteins is the mostly studied and is also the most important class of anti-apoptotic proteins (8. Pavlína Majtnerová, Tomáš Roušar 2018;9. Rathore R et al., 2017). HuR can bind to the adenylate-uridylylate-rich element (ARE) fragment of the mRNA 3'-untranslated region (3'UTR) of various genes (9. Rathore R et al., 2017). The mRNA encoding the cyclinD1 has been experimentally shown to contain an ARE fragment, and HuR may enhance the stability of the factor mRNA by binding to ARE, thereby upregulating the expression of the above factor protein and exerting the corresponding biological effects. Therefore, the present study employed human bladder cancer T24 cells to explore the effect of HuR on the behavior of bladder cancer cells and the relationship between the expression of HuR and CyclinD1and Bcl-2.

Materials & 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. RPMI-1640 cell culture medium, trypsin, fetal bovine serum, 1% streptomycin (Gibco; Thermo Fisher Scientific, Inc.); pU6gRNACas9EGFP vector and pIRES2-ZsGreen vector (Addgene, Inc); Lipofectamine 2000TM transfection reagent, TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.); reverse transcription (RT)-PCR kit, T4 DNA ligase, restriction endonuclease Xho I, EcoR I (Takara Biotechnology, Co., Ltd.); DNA gel recovery kit (Sangon Biotech, Co., Ltd.); Transwell Chamber (Corning, Incorporated); RIPA Lysate, BCA Protein Concentration Kit, MTT Test Kit (Beyotime, Institute of Biotechnology); Apoptosis Detection Kit (Nanjing KeyGEN BioTECH, Co., Ltd.); HuR antibody, cyclinD1 antibody, BCL-2 antibody (Abcam); and the GAPDH horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (ProteinTech Group, Inc.). This project was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Cell culture. T24 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% streptomycin, and grown at 37°C and, 5% CO₂. The cells were routinely subcultured when they reached ~80% confluence.

Construction of the HuR overexpression vector. The total RNA of T24 cells was extracted according to the TRIzol kit instructions, cDNA was obtained by RT-PCR, and PCR was performed using this cDNA as a template: Premix Taq 12.5 µl, 1 µl of upstream and downstream primers, 2 µl of template, and ddH₂O added to a 25 µl of reaction system. The thermocycling conditions were 94 °C for 5 min, 30 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 2 min, and then a 72°C 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 EcoR 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 gRNA of the HuR gene (sequence: 5'-AGAGCGATCAACACGCTGAA-3'; synthesized by Shanghai GenePharma Co., Ltd) was designed using the 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 E. coli DH5 α , 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, cyclinD1 and Bcl-2. After 48 hours of transfection, the control group, HuR overexpression groups and HuR knockdown group were collected, and the total RNA was extracted according to the TRIzol kit instructions. The cDNA was obtained by RT-PCR, and the cDNA was used as a template for qPCR. GAPDH was selected as the internal reference, and the relative expression of HuR, cyclinD1 and Bcl-2 mRNA was determined by the $2^{-\Delta\Delta C_q}$ relative quantitative method. The primer sequences are as presented in Table 1.

Western blot of analysis of HuR, cyclinD1 and Bcl-2. The cells of control group, HuR overexpression group and HuR knockdown groups were collected after 48 h. The cell lysate was lysed at 4 °C, 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% SDS-PAGE was conducted for 2.5 h. After proteins were transferred to the membrane, the negative control membrane was washed in TBS. Membranes were then blocked with 5% skim milk for 1 h at room temperature. Antibodies against HuR, cyclinD1 and Bcl-2 were incubated at 4°C overnight and then membranes were washed for 10 min repeatedly for 3 times in TBST. Secondary antibodies were incubated for 1 h at room temperature, and then washed with TBST for 10 min 3 times. Then the membrane was visualized via exposure development. Gray value analysis was performed using BIO-RAD Image Lab software.

MTT assay for cell proliferation activity. The MTT assay was performed 48 h after cell transfection. The control group, the HuR overexpression group, and the HuR knockdown groups were seeded in a 96-well plate. A total of 10 μ l of MTT was added to each well. After incubating at 37°C for 4 h, the medium was aspirated, and then 150 μ l of DMSO was added and shaken for

10 min. The absorbance value of the optical density (OD) of each well at a wavelength of 568 nm was measured with a microplate reader.

Apoptosis detection via Annexin V-APC/7-AAD double staining. The cells of the control, HuR overexpression group and HuR knockdown groups were collected at 48 h after transfection and then washed 2-3 times with pre-cooled PBS. Cells were adjusted to a cell concentration of $5 \times 10^5/\text{ml}$, and resuspended via of the addition of 100 μl Binding Buffer. Then 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.

Transwell assay 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 $2 \times 10^5/\text{ml}$ with serum-free medium. A total of 800 μl of 10% FBS-containing medium was added to a 24-well plate and placed in a transwell chamber. Then 200 μl of each cell suspension was inserted into the transwell chamber, and cultured at 37°C in a 5% CO_2 incubator for 48 h. The transwell insert was removed, the chamber was carefully washed once 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 wiped clean with a clean cotton ball. Pictures were taken under a microscope and the number of cells was counted.

Statistical analysis. All experiments were repeated at least three times. Statistical analysis was performed using SPSS 23.0 statistical software (SPSS, Inc.) Data were expressed as the mean \pm standard deviation, and an ANOVA and a post hoc test for multiple comparisons was used for comparisons between groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Construction of the HuR overexpression vector and knockdown vectors. As shown in Fig. 1A and Fig.1B, 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 were successfully constructed.

Silencing and overexpression of HuR in response to HuR transfection and HuR knockdown by cas9. The protein expression levels of HuR was detected by western blotting as presented in Fig. 2A. Compared with the control group, the expression of HuR in the HuR overexpression group was significantly increased and decreased in the HuR knockdown group ($F=55.890$, $P=0.001$). The relative expression levels of mRNA of HuR, was detected by PCR (Fig. 2B). Compared with the control group, the expression of HuR was significantly increased in the HuR overexpressing group and decreased in the HuR knockdown group ($F=124.230$, $P=0.001$).

HuR promotes the proliferation of T24 cells. Compared with the control group, the cell viability after 48 h of HuR overexpressing cells was significantly increased and decreased in the HuR knockdown group as presented in Fig. 3 ($F=1929.061$, $P=0.001$).

HuR promotes the migration of T24 cells. Compared with the control group, the number of migrated of HuR overexpressing cells was significantly increased and decreased in the HuR knockdown group as shown in Fig. 4 ($F=145.982$, $P=0.001$).

HuR inhibits T24 cell apoptosis. Compared with the control group, the apoptotic rate of the HuR overexpression group was significantly decreased and increased in the knockdown group (Fig. 5; ($F=665.452$, $P=0.001$).

Effect of HuR on the expression of cyclinD1 and Bcl-2. The expression of protein of cyclinD1 and Bcl-2 were detected by western blotting as presented in Fig. 6A. Compared with the control group, the expression of cyclinD1($F=49.102.762$, $P=0.001$) and Bcl-2 ($F=265.736$, $P=0.001$) in the HuR overexpression group was significantly increased and decreased in the HuR knockdown group.

The relative expression levels of mRNA of cyclinD1 and Bcl-2 were detected by PCR (Fig. 6B). Compared with the control group, the expression of cyclinD1($F=225.681$, $P=0.000$) and

Bcl-2 ($F=202.762$, $P=0.000$) was significantly increased in the HuR overexpressing group and 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 level, transcriptional level and post-transcriptional levels (10. Deng N, Zhou H, Fan H 2017;11. James E, Jenkins T G 2018;12. Rybstein M D et al., 2018). 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 of RBP (13. DeMicco A 2015;14. Peng SS et al., 1998;15. Mukherjee N et al., 2011). HuR is a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, located at 19p13.2, which encodes a variety of proto-oncoproteins (13. DeMicco A 2015;14. Peng SS et al., 1998;15. Mukherjee N et al., 2011). HuR 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 (16. Kim I et al., 2012; 17. Sun D Q et al., 2012). Young et al (18. Young L E et al., 2009) reported that HuR was overexpressed in colon tumor tissues to significantly higher levels than in normal colon tissue, and the increased expression of HuR promoted the development of colon cancer (18. Young L E et al., 2009). Heinonen et al [19,20] demonstrated that in breast cancer patients with non-BRCA1/2 (breast cancer susceptibility gene 1/2, BRCA1/2) 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 metastasis (19. Heinonen M et al., 2011; 20. Heinonen M et al., 2007). The experimental results also suggested that HuR may be involved in lymph node metastasis (19. Heinonen M et al., 2011; 20. Heinonen M et al., 2007). A recent study revealed that HuR was highly expressed in various tumor tissues such as breast, gastric, esophageal and ovarian cancers (21 Danilin S et al., 2010). In the present study, the proliferation rate and migratory ability of HuR overexpressing 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. It was indicated that HuR promoted the proliferation and migration and decreased the apoptosis of human bladder cancer T24 cells.

The cyclinD1 gene is a proto-oncogene that plays a very important regulatory role in the cell cycle of eukaryotic cells (22. Casimiro MC et al., 2017; 23. Ju X et al., 2016). CyclinD1 binds and activates 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 E2F transcription factor; in turn, the E2F transcription factor initiates 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 (22. Casimiro MC et al., 2017; 23. Ju X et al., 2016). The expression of cyclinD1 was significantly increased in many tumor tissues, such as breast cancer, nasopharyngeal carcinoma, lung cancer and liver cancer (24. Bartkova J et al., 2010; 25. Hong J I, Xia H E, Cheng S Q 2010; 26. Pandey A et al., 2018; 27. Huang C Z et al., 2013).

In the present study, the expression of cyclinD1 was significantly increased in the HuR overexpressing group and significantly decreased in the HuR knockdown group, suggesting that the mechanism of HuR on biological behavior of T24 cells may be related to the expression of cyclinD1 and 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 is highly expressed, it inhibits the occurrence of apoptosis, whereas when Bcl-2 expression is low, apoptosis is promoted. The present study revealed that when compared with 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, suggesting that HuR inhibits the apoptosis of human bladder cancer T24 cells, which is consistent with the conclusion that HuR inhibits apoptosis.

Conclusions

In conclusion, RNA-binding proteins HuR promotes proliferation and migration of bladder cancer T24 cells and inhibits apoptosis, and the mechanism may be related to influencing cell

cycle and apoptosis. And the expression of HuR is related to cyclinD1 and apoptosis-related proteins Bcl-2. Therefore, the HuR gene may be a new target gene of target therapy for bladder cancer. However, there are some limits in this study. Such as only one bladder cancer cell line was applied in this study. And experiments including animal model and human tissue samples were not carried out as well. And meanwhile due to shortage of finance, only one apoptosis-related protein was selected in this experiment. So, further investigation especially in vivo studies are still needed in future.

Acknowledgements

Kewen Zheng, Xiaomin Han, Yan Su and Qiang Ma conceived and designed the experiments. Kewen Zheng, Xiaomin Han and Qiang Ma performed the experiments and wrote the paper. Kewen Zheng, and Yan Su analyzed the data. Kewen Zheng performed the statistical analyses. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy and integrity of any part of the work are appropriately investigated and resolved.

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Table 1 (on next page)

Table

Table 1. Primer sequences of HuR, cyclinD1, Bcl-2 and GAPGH

1 Table 1. Primer sequences of HuR, cyclinD1, Bcl-2 and GAPGH

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

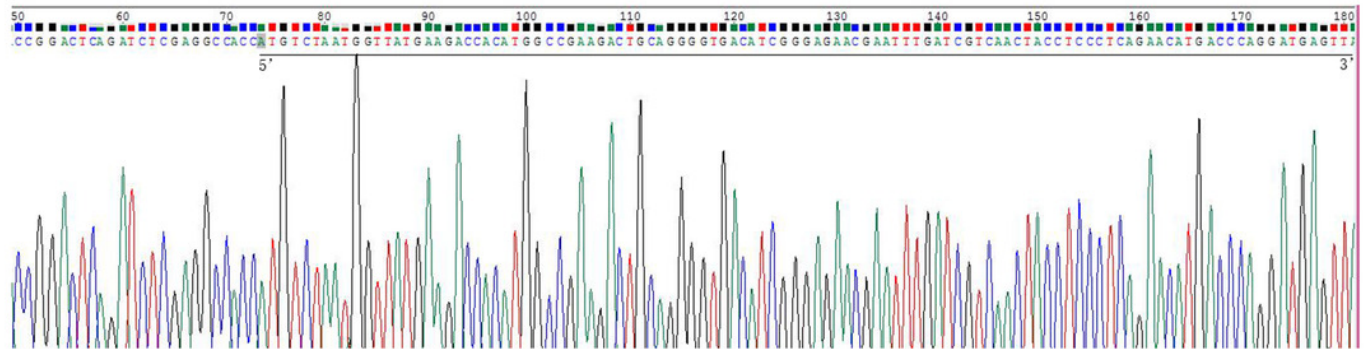
2

Figure 1

Figure 1. Partial sequence of HuR overexpression and knockdown plasmid vector

Figure 1A. Partial sequence of HuR overexpression plasmid vector Note: Sequence underlined is inserted HuR sequence, and sequence ununderlined is vector backbone
Figure 1B. Partial sequence of HuR knockdown plasmid vector Note: Sequence underlined is inserted HuR sequence and sequence ununderlined is vector backbone

A



B

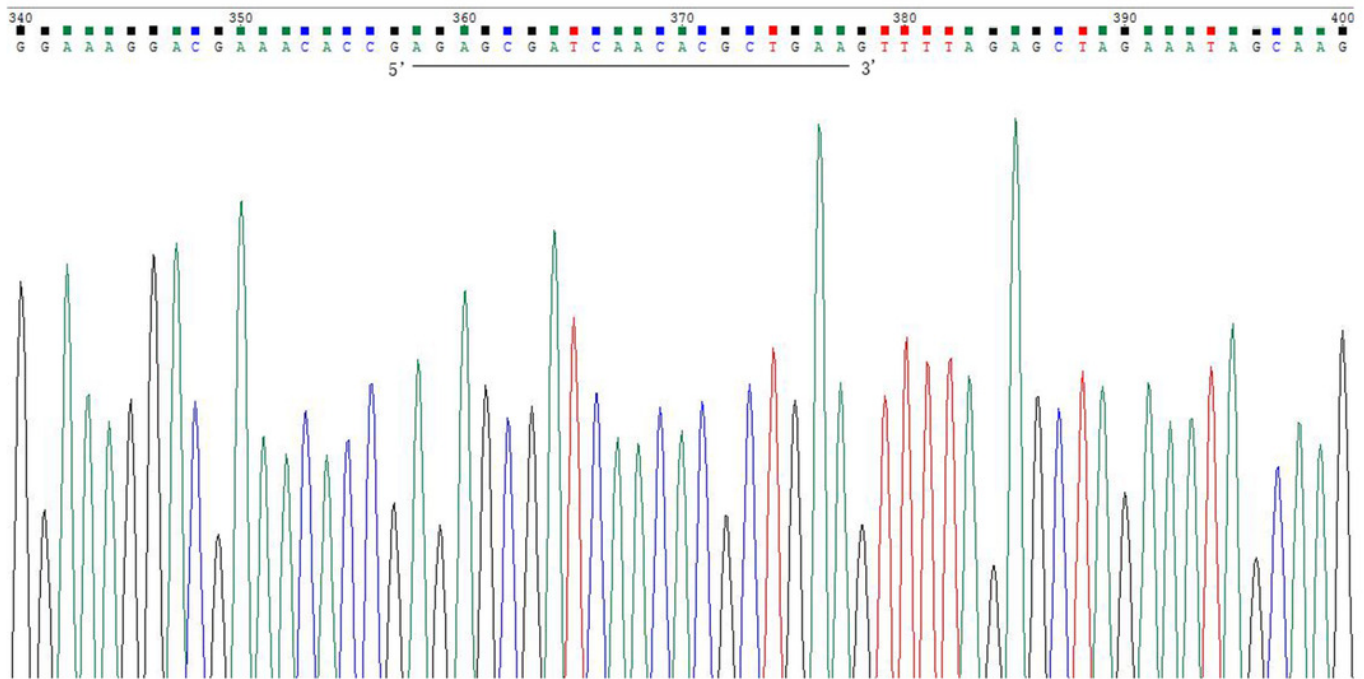


Figure 2

Figure 2. Silencing and overexpression of HuR in response to HuR transfection and HuR knockdown by cas9

Figure 2A is expression of HuR protein detected by western blot. The protein expression of HuR in the overexpression group was significantly increased and decreased in the knockdown group. Figure 2B is expression of HuR mRNA detected by RT-qPCR. The expression levels of mRNA of HuR was significantly increased in the overexpressing group and decreased in the knockdown group. * $P < 0.05$. Control, non-transfected cells; overExp-HuR, the HuR overexpression group; cas9-HuR, the HuR knockdown group; HuR, human antigen R. Note. (1) the original data were normal distributed; (2) the original data were compared quantitatively in each group.

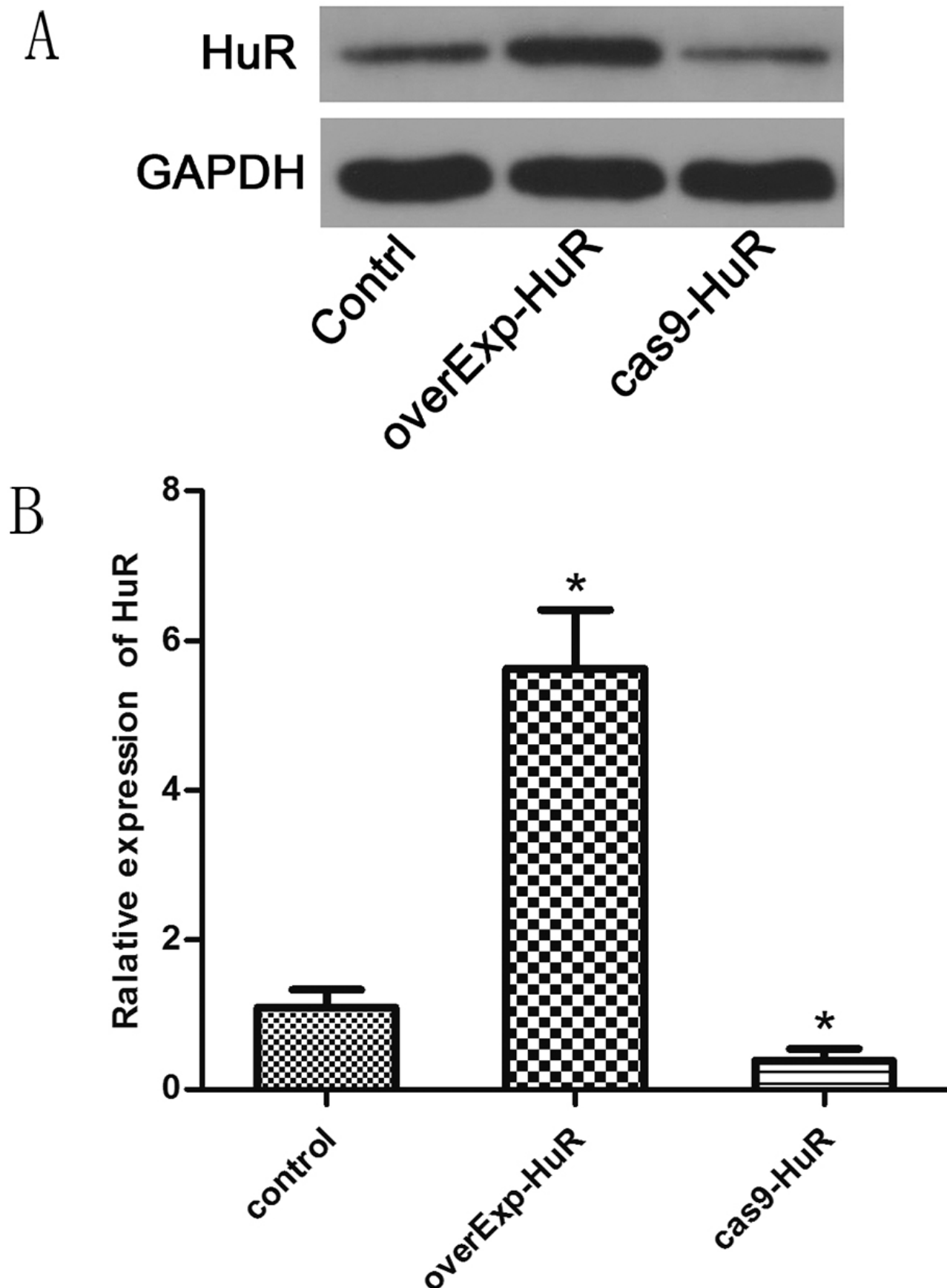


Figure 3

Figure 3. Effect of HuR on the proliferation of T24 cells.

The cell viability after 48 h of HuR overexpressing cells was significantly increased, and decreased in HuR knockdown group. * $P < 0.05$. Control, non-transfected cells; overExp-HuR, the HuR overexpression group; cas9-HuR, the HuR knockdown group; HuR, human antigen R. Note. (1) the original data were normal distributed; (2) the original data were compared quantitatively in each group.

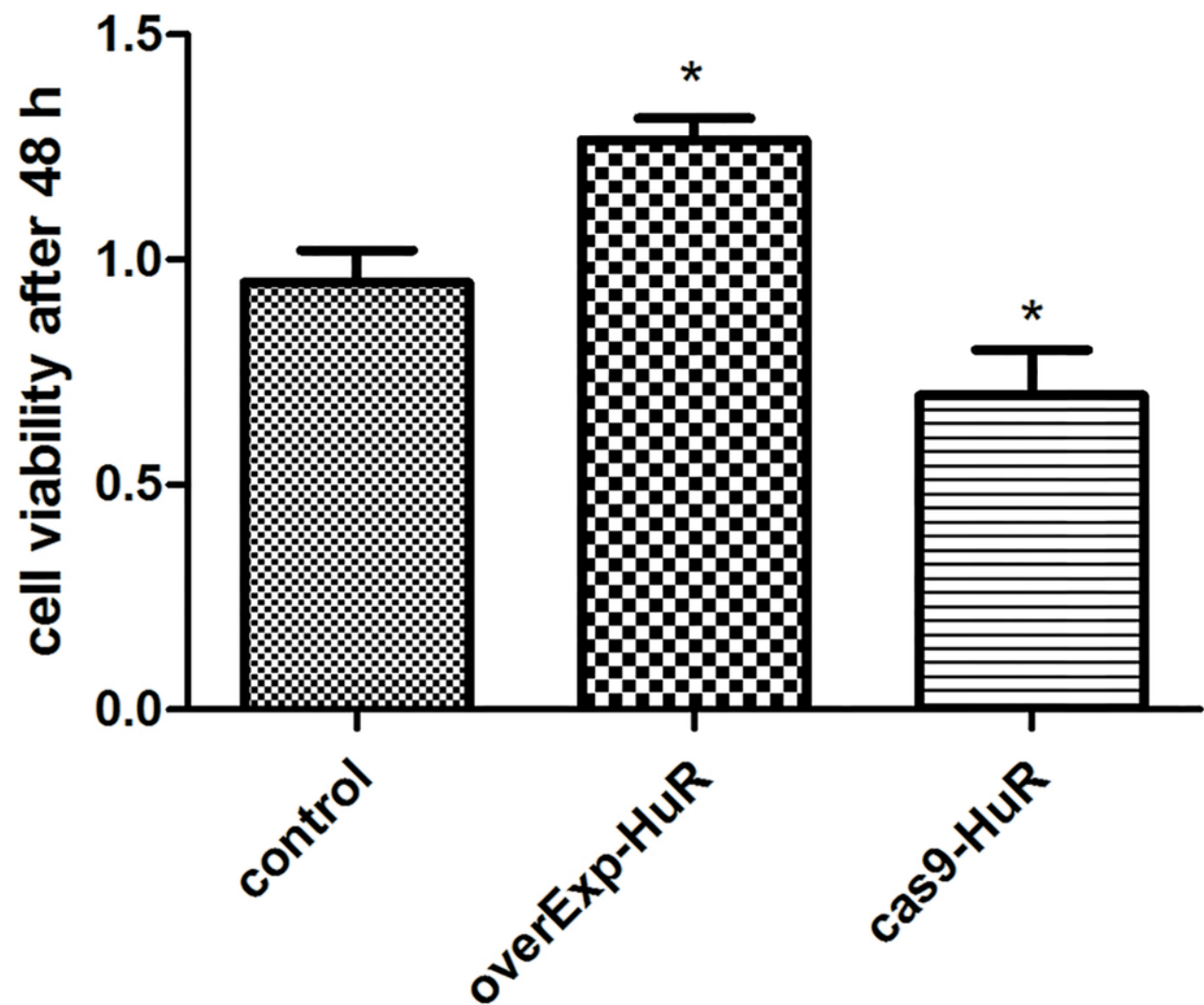


Figure 4

Figure 4. Effect of HuR on T24 cell migration.

Cell migration of the (A) Control, (B) overExp-HuR and (C) cas9-HuR groups. (D) Number of migrating cells in each group. The number of migrated of HuR overexpressing cells was significantly increased and decreased in the knockdown group. $P < 0.05$. Control, non-transfected cells; overExp-HuR, the HuR overexpression group; cas9-HuR, the HuR knockdown group; HuR, human antigen R. Note. (1) the original data were normal distributed; (2) the original data were compared quantitatively in each group.

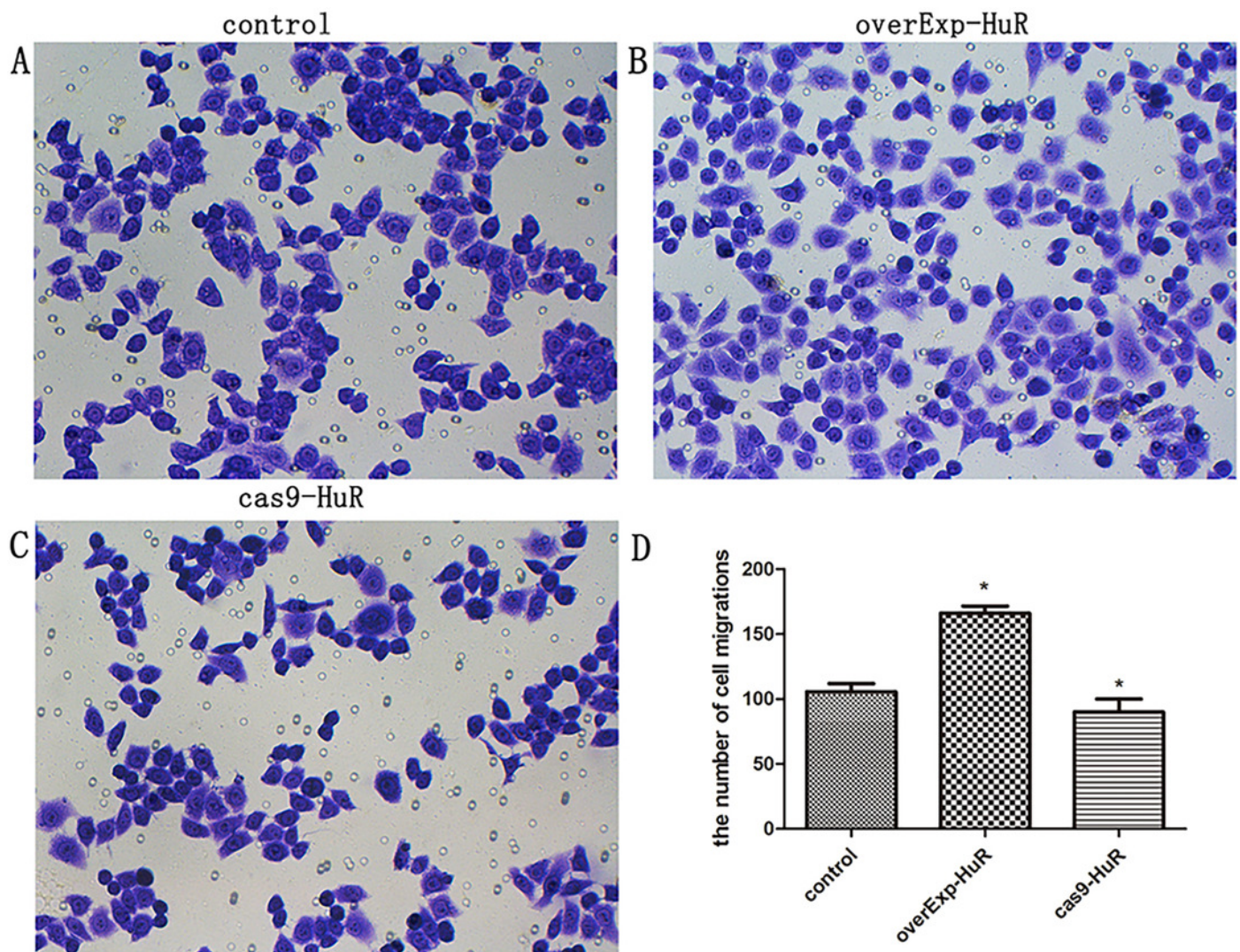


Figure 5

Figure 5. Effect of HuR on the apoptosis of T24 cells.

The apoptotic rate of the HuR overexpression group was significantly decreased and increased in the knockdown group. * $P < 0.05$. Control, non-transfected cells; overExp-HuR, the HuR overexpression group; cas9-HuR, the HuR knockdown group; HuR, human antigen R. Note. (1) the original data were normal distributed; (2) the original data were compared quantitatively in each group.

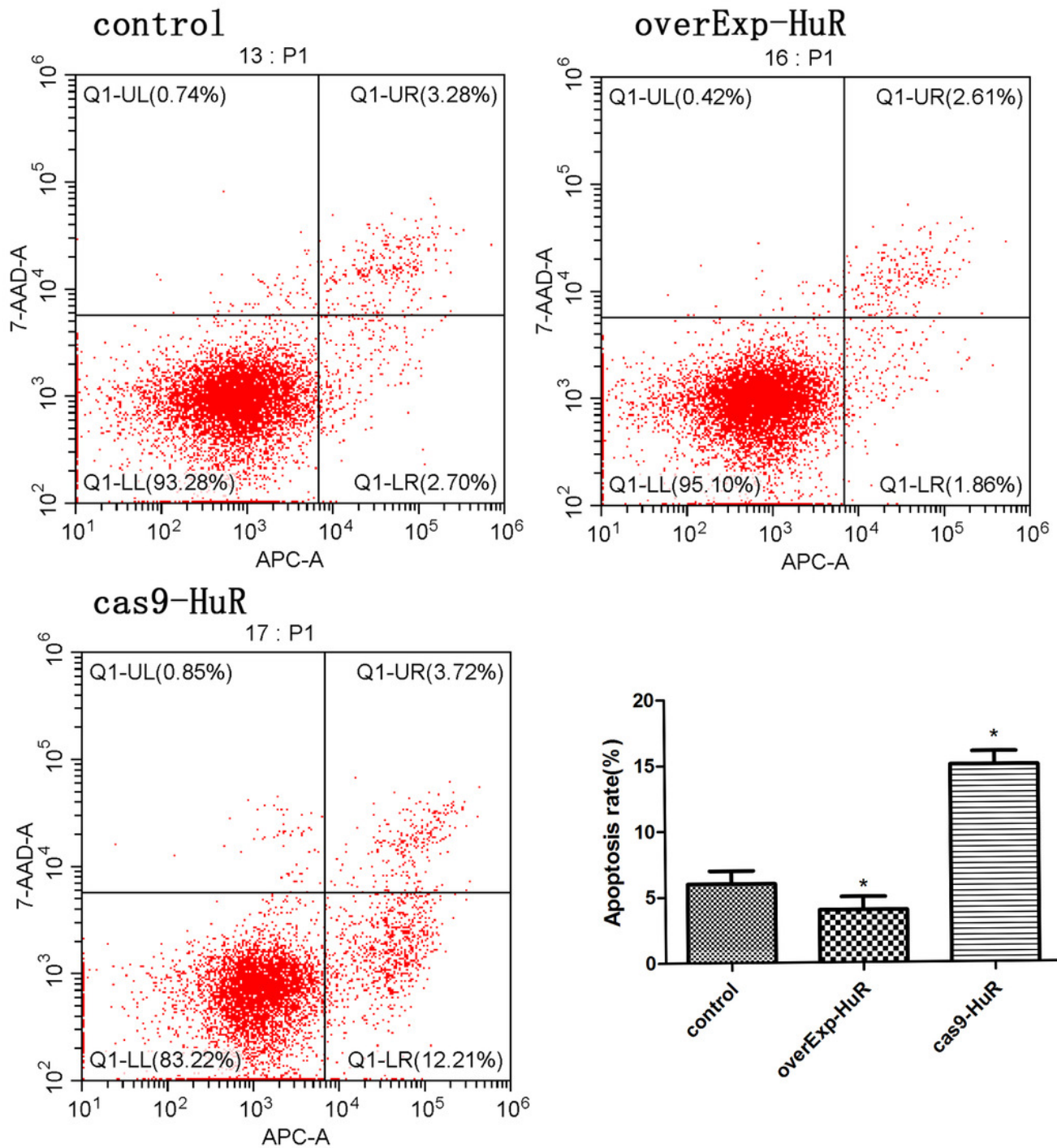


Figure 6

Figure 6. Effect of HuR on cyclinD1 and Bcl-2.

Figure 6A is expression of protein of cyclinD1 and Bcl-2 detected by western blot; Figure 6B is expression of HuR mRNA detected by RT-qPCR. The expression of protein and mRNA of cyclinD1 and Bcl-2 was significantly increased in the HuR overexpression group and decreased in the knockdown group. * $P < 0.05$. Control, non-transfected cells; overExp-HuR, the HuR overexpression group; cas9-HuR, the HuR knockdown group; HuR, human antigen R. Note. (1) the original data were normal distributed; (2) the original data were compared quantitatively in each group.

