Original Paper

MiR-291b-3p Induces Apoptosis in Liver Cell Line NCTC1469 by Reducing the Level of RNA-binding Protein HuR

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Key Words

miR-291b-3p • NCTC1469 cell • Apoptosis • HuR, Bcl-2

Abstract

Background: There is increasing evidence that miRNAs are involved in cellular apoptosis. However, the specific role of miR-291b-3p in apoptosis has not been elucidated. In the present study, we investigated the effect of miR-291b-3p on NCTC1469 cell growth and apoptosis.

Methods: Cell viability and apoptosis were examined in NCTC1469 cells transfected with miR-291b-3p mimics, inhibitor miRNA or negative control. Using computational miRNA target prediction databases, HuR was predicted as a target of miR-291b-3p. Luciferase assay, immunofluorescence and western blot were used to further explore the effects of miR-291b-3p on HuR expression. In addition, the effect of HuR on cell apoptosis was evaluated using a HuR-specific siRNA. **Results:** TNF- α -induced hepatocyte apoptosis was accompanied by enhanced expression of miR-291b-3p, suggesting that miR-291b-3p might contribute to the apoptotic process. Follow-up experiments showed that upregulation of miR-291b-3p decreased cell viability and induced NCTC1469 cell apoptosis. Additionally, similar to the activity of miR-519, which is another member of the same miRNA family, miR-291b-3p suppressed HuR translation through binding to the HuR coding region (CR). We further showed that the downregulation of HuR expression by miR-291b-3p was accompanied by reduced Bcl-2 expression. Moreover, knockdown of HuR also impaired Bcl-2 expression and increased the ratio of Bax/Bcl-2. More significantly, downregulation of miR-291b-3p failed to increase Bcl2 expression in NCTC1469 cells that were co-transfected with siRNA-HuR. Finally, inhibition of miR-291b-3p led to reduced apoptosis, while knockdown of HuR by siRNA promoted apoptosis, even in NCTC1469 cells that were co-transfected with the miR-291b-3p inhibitor. **Conclusion:** The current data suggested that miR-291b-3p contributed to NCTC1469 cell apoptosis by regulating the expression of HuR, which in turn increased Bcl-2 stability.

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Introduction

MiRNAs are small non-coding RNAs that potently regulate gene expression in mammalian cells. The primary transcript of miRNA is initially transcribed by RNA polymerase II or RNA polymerase III. In the nucleus, the primary transcript is processed by Drosha and transformed into the pri-miRNA with approximately 60 to 110 nucleotides. The transcript is then transported to the cytoplasm and further cleaved to the mature form by Dicer. The mature miRNA is incorporated into the RNA-induced silencing complex (RISC) [1]. The complex is tethered to a complementary sequence within the 3'-untranslated region (UTR) of the target mRNA, resulting in the suppression of gene translation and expression. It has been suggested that over 30% of all human genes are regulated by miRNAs, many of which are involved in important cellular pathways [2]. In recent years, numerous investigations have implicated miRNA dysregulation during cellular apoptosis, leading to additional studies on the involvement of miRNAs and their target genes in apoptotic pathways. For instance, miR-150 and miR-630 were shown to induce pancreatic cancer cell apoptosis by targeting IGF-1R, and miR-21 was shown to have anti-apoptotic functions by targeting pro-apoptotic genes, such as FasL, PTEN and PDCD4 [3, 4].

In addition to miRNAs, AU-rich elements (AREs)-mediated transcript degradation is considered as another important gene regulation mechanism at the post-transcriptional level [5]. Undoubtedly, AREs are important cis-elements that control cell growth and proliferation because a number of cancer-related transcripts contain AREs in the 5'-untranslated region (UTR) or 3'-UTR, such as cytokines, growth factors and invasion factors [6]. AREs are recognized by RNA binding proteins, which then affect gene expression by changing the stability and translation efficiency of mRNAs [6]. The human embryonic lethal abnormal vision-like (ELAV) protein HuR is an RNA binding protein in the Hu family [7]. It is widely expressed in mammalian cells and can bind to AREs to adjust mRNA stability.

According to the number and distribution of AUUUA nucleotides, AREs can be divided into three types [8]. Type I contains several copies of the AUUUA motif; type II has at least two copies of UUAUUUA (U/A) (U/A); and type III lacks the typical AUUUA motif in the U-rich regions. HuR can recognize and bind to all three types of AREs in the 5'-UTR or 3'-UTR of mRNAs. For most of these mRNAs, HuR was reported to increase their stability [9-11]. It has also been shown that, while HuR is predominantly localized in the nucleus, it can be shuttled between the nucleus and the cytoplasm. This translocation allows HuR to efficiently stabilize its target mRNAs [12]. Over the past decades, there has been increasing evidence suggesting that HuR plays a key role in carcinogenesis and cancer progression by regulating the expression of numerous target genes, such as p53, p21, p27, and Bcl-2 [13-15].

Recently, it was reported that many miRNAs, such as miR-9, miR-34a, miR-16, miR-125a, miR-29a, miR-200c and miR-519, can regulate the protein expression of HuR by binding to specific regions in the 3'UTR or 5'UTR of HuR [16-21]. The present study describes a novel miRNA, miR-291b-3p, that participates in HuR regulation. Furthermore, our study shows that inhibition of HuR expression by miR-291b-3p in NCTC1469 cells may induce apoptosis through downregulation of Bcl-2.

Materials and Methods

Cell culture

Murine liver cell line NCTC1469 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Horse serum (Atlanta Biolabs), 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc.), at 37°C in a humidified atmosphere with 5% CO₂.

Isolation of mouse primary hepatocytes

Male C57BL/6J mice (12 weeks old) were provided by the Peking University Health Science Center (Beijing, China). Mouse primary hepatocytes were isolated as previously described [22].

Transient transfection

Before transfection, 1×10^5 cells per well were seeded in a 6-well plate with 2 ml DMEM culture medium containing serum and antibiotics. Meanwhile, miR-291b-3p mimic, inhibitor, or negative control (Genepharma) was pre-incubated with HiPerFect transfection reagent (QIAGEN) at room temperature for 10 min. The complex was then transfected into the NCTC1469 cells at a final concentration of 50 nM. The transfected cells were incubated under normal growth conditions for another 48 h.

RNA extraction and real-time PCR

Total RNA was extracted from NCTC1469 cells with Trizol (Invitrogen) according to the manufacturer's instructions. The quality of the RNA samples was monitored by analysis of OD_{260}/OD_{280} .

To measure the level of miR-291b-3p, a quantitative 2-step RT-PCR assay was performed using SYBR Green Supermix (Bio-Rad) in a BIO-RAD iCycler iQ real-time PCR detection system.

For the quantitation of miRNA, 1 μ g of the total RNA were reverse transcribed using Taq-Man MicroRNA Reverse Transcription Kit (Applied Biosystems) with specific primers for miR-291b-3p and U6 (Shanghai Sangon Technology), and subsequently the PCR amplifications were performed in 20 μ l reaction volumes containing 10 μ l SYBR Green Supermix, 0.8 μ l forward primer, 0.8 μ l reverse primer, 7.4 μ l ddH₂O and 1 μ l template cDNA. The thermal cycling conditions were a hot start step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The relative level of miR-291b-3p was determined using the $2^{-\Delta\Delta Ct}$ analysis method, where U6 was used as the endogenous control. Nucleotide primers used for reverse transcription were as follows (5'-3'): miR-291b-3p, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAAAC; U6, GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAATATG.

The primers used for real-time PCR were as follows (5'-3'): miR-291b-3p forward, GCAAAGTGCATCCATTTTGTGTGT; U6 forward, GCGCGTCGTGAAGCGTTC; Universal reverse primer, GTGCAGGGTCCGAGGT.

Protein extraction and western blot

Proteins were extracted from NCTC1469 cells with RIPA buffer (1% TritonX-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 10 mmol/L Tris-HCl (pH 7.0)) (Solarbio, China) supplemented with a protease inhibitor cocktail (Sigma). The cell lysates were separated by 10% SDS-PAGE and transferred electrophoretically onto PVDF membranes. After blocking with 8% milk in PBS (pH 7.5), the membranes were incubated with the following specific primary antibodies overnight: anti-HuR, anti-bcl-2, anti-bax and anti- β -actin (SantaCruz Biotechnology, Inc.). The appropriate HRP-conjugated anti-rabbit IgG secondary antibody (Abmart, all at 1:5000) was subsequently applied, and immunodetection was performed using the ECL Plus detection system (Millipore) according to the manufacturer's instructions. β -actin was used as the internal control.

Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay

To explore the effect of miR-291b-3p on cell viability, 5,000 cells per well in a 100 μ l medium were seeded in 96-well plates and transfected with miR-291b-3p mimics (50 nM) or negative control-miRNA mimics (50 nM), as described above. Every 24 h after transfection, 20 μ l of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Solarbio, China) was added to wells and were incubated with the cells for 4 h. After removing the medium, the blue formazan was dissolved with 200 μ l dimethyl sulfoxide (DMSO) and the absorbance was measured at 550 nm. Wells containing only NCTC1469 cells served as blanks.

Hoechst 33258 staining

NCTC1469 cells were cultured in 6-well plates. After 48 h transfection with miR-291b-3p mimics, inhibitors or negative control, cells were washed with PBS and stained with Hoechst 33258 (10 μ g/ml) for 5 min before being washed three times with PBS.

Immunofluorescence

NCTC1469 cells were cultured on 6-well chamber slides and fixed with 4% paraformaldehyde for 10 min at -20°C. The slides were washed in PBS for three times, and incubated with a polyclonal antibody against HuR (1:50 diluted in PBS with 1% BSA, 50 μ l/slide) for 2 h at room temperature. After washing with PBS for three times (5 min per time), the slides were incubated with TRITC-conjugated anti-mouse

IgG (1:100 diluted in PBS with 1% BSA, 50 µl/slide) for 1 h at room temperature. Three times after washing the slides in PBS, the slides were incubated with Hoechst 33258 (10 µg/ml) for 5 min. Then, the slides were washed again and examined using a fluorescence microscope.

Quantification of apoptotic cells

To quantify apoptotic cells, flow cytometry was performed with an Annexin V-fluorescein-5-isothiocyanate apoptosis detection kit (Bio-vision, USA). 48 h after transfection with miR-291b-3p mimics (50 nM) and a negative control (50 nM), NCTC1469 cells were harvested in a 5 ml tube. Then, the cells were washed with cold PBS and resuspended at a final concentration of 1×10^6 cells/ml. FITC-AnnexinV (5 µl) and propidium iodide were gently mixed and incubated with the cells for 15 min at a room temperature. After incubation, the samples were analyzed by flow cytometry within 1 h.

Luciferase target assay

For luciferase assay, the 3' untranslated region (UTR) or coding region of HuR including the binding sites for miR-291b-3p was amplified from NCTC1469 cells by using the following primers (restriction sites are underlined):

HuR-CD-N-XhoI 5' CCCTCGAG CTGGCTCTGGAATCATTTGCT

HuR -CD-C-XhoI 5' CCCTCGAG AGGCAGTCTTCGGTTCTTGA

HuR-UTR-N-XhoI 5' CCCTCGAG CCTATATGGGGTTGCTTCCA

HuR-UTR-C-XhoI 5' CCCTCGAG CCAACCAGCCTTCTTTTCTG

PCR was performed with genome DNA isolated from NCTC1469 cells and the PCR product was then digested with XhoI (NEB). Then, the fragment was inserted into the XhoI-linearized pGL3 (Promega) luciferase reporter vector. To ensure the ligation efficiency, both the PCR products and the XhoI-linearized pGL3 vectors were treated with thermosensitive alkaline phosphatase (NEB) at 80 °C for 20 min. Details of PCR procedures are described as follows: a hot start step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 55 °C for 45 s, 72 °C for 30 s.

To conduct luciferase reporter assay, 5000 cells per well in a 100 µl medium were seeded in 96-well plates. After incubation overnight, the cells were transfected with the modified firefly luciferase vector (500 ng/µl) with lipofectamine TM 2000 Reagent (Invitrogen) according to the manufacturer's instruction. 48 h after transfection, the firefly and renilla luciferase activities were measured with the Dual-luciferase reporter assay system (Promega). To control the transfection efficiency, Firefly activity was normalized to renilla activity.

Inhibition of HuR by RNA interference

HuR-specific siRNA (siHuR) and negative control were purchased from Genepharma. 1×10^5 cells per well in a 6-well plate were transfected with 50 nM siHuR or negative control for 48 h using HiperFect transfection reagent (QIAGEN) as described above.

Statistical analysis

Data were presented as mean $\pm$ SE from 3 independent experiments. Statistical analysis was carried out with Student's *t* test. $P < 0.05$ was considered as statistically significant difference.

Results

TNF- α induced hepatocyte apoptosis, accompanied by enhanced level of miR-291b-3p

In previous study, we analyzed miRNAs in the livers of db/db mice ($n=5$) and control mice ($n=5$) by miRNA microarray and verified by real-time PCR. The results showed that increased level of miR-291b-3p in the livers was accompanied by enhanced TNF- α level in the serum of db/db mice (data not shown). In the present study, to identify the impact of miR-291b-3p on cell apoptosis induced by TNF- α , we extended the observation in the db/db mice to murine liver cell line NCTC1469. To investigate the effect of TNF- α on hepatocyte apoptosis, murine liver cell line NCTC1469 was treated with 20 ng/ml TNF- α for 48 h. Hoechst staining showed that TNF- α significantly induced apoptosis in these cells (Fig 1. A).

Fig. 1. TNF- α induced hepatocyte apoptosis, accompanied by enhanced level of miR-291b-3p. Murine liver cell line NCTC1469 was treated with 20 ng/ml TNF- α for 48 h. Hoechst staining showed that TNF- α significantly induced NCTC1469 cell apoptosis (A). White arrow indicated apoptotic cells. The level of miR-291b-3p was increased in the NCTC1469 cells (B) and the primary hepatic cells (C) treated with TNF- α compared with the negative control. Data represent the means $\pm$ SEM, n=3 independent experiments. ** p <0.01 versus control.

Fig. 2. MiR-291b-3p regulated NCTC1469 cell viability. NCTC1469 cells were transfected with miR-291 mimics, inhibitor or negative control for 24, 48, 72 h. Upregulation of miR-291b-3p decreased cell viability by 25% and 30% at 48 h and 72 h (A), while downregulation of miR-291b-3p enhanced cell viability in the NCTC1469 cells by 20% and 30% at 48 h and 72 h, respectively (B), as analyzed by MTT assay. Data represent the means $\pm$ SEM, n=6 independent experiments. * p <0.05 versus control.

Moreover, we also explored the effect of TNF- α on the expression of miR-291b-3p in both NCTC1469 cells and primary hepatic cells. Our data indicated that the TNF- α treatment significantly increased the level of miR-291b-3p in both cell populations (Fig 1. B and C). These *in vitro* observations indicated that miR-291b-3p might contribute to TNF- α -induced apoptosis of hepatocytes.

MiR-291b-3p regulated NCTC1469 cell viability

To determine the effect of miR-291b-3p on cell viability, NCTC1469 cells were transfected with miR-291 mimics, inhibitor or negative control for 24, 48, and 72 h. As shown in Fig 2. A, upregulation of miR-291b-3p by the mimics decreased cell viability by 25% and 30% at 48 h and 72 h, respectively, while downregulation of miR-291b-3p by the inhibitor enhanced cell viability by 20% and 30% at 48 h and 72 h, respectively (Fig 2. B). These results demonstrated that miR-291b-3p could modulate NCTC1469 cell viability.

Upregulation of miR-291b-3p induced NCTC1469 cell apoptosis

We next observed that upregulation of miR-291b-3p enhanced cell apoptosis by nearly fourfold versus the negative control in NCTC1469 cells (Fig 3. A). Hoechst 33342 staining

Fig. 3. Upregulation of miR-291b-3p induced NCTC1469 cell apoptosis. MiR-291b-3p mimics or inhibitor were transfected into NCTC1469 cells for 48 h. Upregulation of miR-291b-3p enhanced cell apoptosis by 130% versus negative control in the NCTC1469 cells (A), as tested by Annexin V and PI kit. (B) The apoptotic cells increased in the NCTC1469 cells transfected with miR-291b-3p mimics, as examined by Hoechst 33342 staining. White arrow indicated apoptotic cells. As assessed by Western blot, upregulation of miR-291b-3p could decrease the level of Bcl2 and increase the levels of Bax and Bim (C), while suppression of miR-291b-3p could elevate the expression level of Bcl-2, and reduced the levels of Bax and Bim (D). Data represent the means $\pm$ SEM, n=3 independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control.

also verified that the number of apoptotic cells were higher in the NCTC1469 cells that were transfected with miR-291b-3p mimics when compared with the negative control (Fig 3. B). Together these data indicated that upregulation of miR-291b-3p induced NCTC1469 cell apoptosis. Furthermore, western blot analysis of apoptotic protein expression showed that upregulation of miR-291b-3p resulted in lower expression of Bcl2 and higher expression of Bax and Bim (Fig 3. C). Moreover, suppression of miR-291b-3p with the specific inhibitor restored the expression of Bcl-2 and reduced the levels of Bax and Bim (Fig 3. D). Taken together, these results indicated that miR-291b-3p might induce NCTC1469 cell apoptosis partially through the regulation of Bcl-2, Bax and Bim expression.

MiR-291b-3p directly bound to the coding region of HuR

MiR-291b-3p belongs to the same family of miRNAs that includes miR-519b-3p, miR-519c-3p, and miR-519a. In previous studies, it has been shown that miR-519 reduces cell proliferation by directly regulating HuR expression [21, 23]. Thus, the sequences of HuR from *Mus musculus* and *Homo sapiens* were aligned. As shown in Fig 4. A, the nucleotides in the coding region (CR) of HuR from 902 bp to 923 bp in *Mus musculus* and from 886 bp to 907

Fig. 4. MiR-291b-3p directly bound to the coding region of HuR. The sequences of HuR from *Mus musculus* and *Homo sapiens* were aligned. The nucleotides in the coding region (CR) of HuR from 902 bp to 923 bp in *Mus musculus* were highly conserved with the nucleotides from 886 bp to 907 bp in *Homo sapiens*. Poorly conserved amino acids of HuR in the 3'-untranslated region were defined in *Mus musculus* and *Homo sapiens*. The sequence of miR-291b-3p with miR-519b-3p, miR-519c-3p, and miR-519a was also aligned (A). The sequence of miR-291b-3p was highly consistent with miR-519b-3p, miR-519c-3p, and miR-519a (B). MiR-291b-3p from mouse was aligned with the murine HuR mRNA (C). As confirmed by luciferase reporter assay, overexpression of miR-291b-3p could significantly reduce the luciferase activity in the HEK 293T cells transfected with luciferase reporter vector with the HuR-CR region, while the luciferase activity was slightly reduced when HEK cells were co-transfected with luciferase reporter vector containing the HuR-3'-UTR and miR-291b-3p mimics (D). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$ versus control.

bp in *Homo sapiens* were highly conserved, and this sequence was confirmed as the target site of miR-519. Poorly conserved amino acids in the 3'-untranslated region of HuR were also defined in *Mus musculus* and *Homo sapiens*. Furthermore, we aligned the sequence of miR-291b-3p with those of miR-519b-3p, miR-519c-3p, and miR-519a. Fig. 4B shows that there is high similarity between the four miRNA sequences. Based on the sequence analysis above, murine miR-291b-3p was then aligned with the murine HuR mRNA. The result indicated that HuR may be a potential target gene of miR-291b-3p (Fig. 4. C). A luciferase reporter assay was then used to assess whether miR-291b-3p can directly bind to the HuR mRNA. Overexpression of miR-291b-3p significantly reduced the luciferase activity in HEK 293T cells that were transfected with the luciferase reporter vector containing the HuR-CR region. However, the luciferase activity was only slightly reduced when HEK cells were co-transfected with both the luciferase reporter vector containing the HuR-3'-UTR and the miR-291b-3p mimics (Fig. 4. D). These data suggest that miR-291b-3p directly modulates HuR expression by binding to the coding region of HuR.

Additional validation of HuR modulation by miR-291b-3p

To further confirm that HuR is negatively regulated by miR-291b-3p, HuR expression was analyzed by immunofluorescence. We found that HuR was predominantly located in

Fig. 5. HuR is the host gene of miR-291b-3p. Immunofluorescence revealed that HuR was predominately located in the nucleus and significantly reduced in the NCTC1469 cells transfected with miR-291b-3p mimics for 48 h (A). As analyzed by Western blot, overexpression of miR-291b-3p reduced the protein level of HuR (B), while suppression of miR-291b-3p enhanced HuR level (C). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

Fig. 6. Suppression of HuR expression could induce apoptosis of NCTC1469 cells. The levels of HuR and Bcl-2 were reduced in the NCTC1469 cells transfected with siRNA of HuR for 48 h, while the Bax expression was not changed (A). When HuR expression was downregulated, cell apoptosis was increased by 123% in comparison with the control group, as analyzed by flow cytometry (B). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p<0.05$, ** $p<0.01$ versus control.

the nucleus 48 h after the transfection of miR-291b-3p mimics, and the level of HuR was significantly reduced compared with the negative control (Fig 5. A). Furthermore, western blot analysis of HuR expression in cells that were transfected with the miR-291b-3p mimics or inhibitor for 48 h revealed that overexpression of miR-291b-3p reduced the protein level of HuR (Fig 5. B), while suppression of miR-291b-3p enhanced the level of HuR (Fig 5. C). However, no changes were detected in the mRNA level of HuR under both conditions, indicating that miR-291b-3p affected HuR expression at the post-transcriptional level

Fig. 7. MiR-291b-3p induced cell apoptosis by reducing HuR expression. Western blot showed the levels of HuR and Bcl2 were reduced in the NCTC1469 cells transfected with miR-291b-3p mimics and siRNA-HuR (A). Downregulation of miR-291b-3p elevated the expression of HuR and Bcl2, while downregulation of HuR reduced Bcl-2 expression. More significantly, downregulation of miR-291b-3p could no longer increase the level of Bcl2 in the NCTC1469 cells transfected with siRNA-HuR (B). Downregulation of HuR did not affect the level

of miR-291b-3p in the NCTC1469 cells, as shown by RT-qPCR (C). Downregulation of miR-291b-3p could induce cell apoptosis when NCTC1469 cells were transfected with siRNA of HuR (D). Data represent the means $\pm$ SEM, $n=3$ independent experiments. * $p < 0.05$ versus control. # $p < 0.01$ versus siHuR+miR-291b-3p inhibitors.

(data not shown). These data further confirmed that miR-291b-3p negatively regulates the expression of HuR in NCTC1469 cells.

Suppression of HuR expression induced apoptosis of NCTC1469 cells

Positive regulation of Bcl-2 expression by HuR in various cell types has been previously described [15]. To identify the downstream effects of HuR on cell apoptosis, NCTC1469 cells were transfected with a specific siRNA against HuR. As a result, the HuR expression level was reduced by 75% compared with the negative control. Additionally, the level of Bcl-2 was also reduced while Bax expression was not changed in these cells (Fig 6. A). Given the results shown in Fig 3. C and the fact that miRNAs can have multiple target genes, we reasoned that, when the miR-291b-3p mimics were transfected into the NCTC1469 cells, downregulation of Bax was due to the effect of miR-291b-3p on target genes different from HuR. Flow cytometry

analysis of the apoptosis rate after HuR knockdown revealed an 11.29% increase relative to the control group. These findings indicated that HuR knockdown reduced Bcl-2 expression and enhanced apoptosis in NCTC1469 cells.

MiR-291b-3p induced cell apoptosis by reducing HuR expression

Western blot analysis showed that the levels of HuR and Bcl2 were reduced in NCTC1469 cells transfected with either miR-291b-3p mimics or siRNA-HuR (Fig 7. A). In contrast, downregulation of miR-291b-3p elevated the expression of both HuR and Bcl2, while downregulation of HuR by siRNA alone reduced Bcl-2 expression. More significantly, downregulation of miR-291b-3p failed to induce Bcl2 expression in NCTC1469 cells that were transfected with siRNA-HuR (Fig 7. B). Moreover, RT-qPCR analysis showed that the downregulation of HuR did not affect the level of miR-291b-3p transcript in NCTC1469 cells. In addition, transfection of the miR-291b-3p inhibitor alone led to reduced apoptosis, while suppression of HuR by siRNA promoted apoptosis, even in cells that were co-transfected with the miR-291b-3p inhibitor (Fig 7. D). Taken together, these results indicated that miR-291b-3p promoted NCTC1469 cell apoptosis by reducing HuR expression.

Discussion

Tumor necrosis factor- α (TNF- α) is an important proinflammatory cytokine that is linked to various diseases [24]. In hepatocytes, TNF- α binds to TNF receptor 1 (TNFR1) and causes the activation of cellular signaling pathways that control either cell survival or cell death. Although the mechanism by which TNF- α dictates cell fate is not understood, a high concentration of TNF- α in liver causes significant damage to the tissue, as indicated by obvious hepatocyte apoptosis [25]. In the present study, we showed for the first time that the expression of miR-291b-3p is upregulated during TNF- α -mediated apoptosis of NCTC1469 cells, suggesting that miR-291b-3p plays an important role in the apoptotic process. We then found that upregulation of miR-291b-3p with specific miRNA mimics resulted in decreased cell viability and increased cell apoptosis, while downregulation of miR-291b-3p with an inhibitor had anti-apoptosis and pro-survival effects.

Evidence of apoptosis regulation by miRNAs has been presented previously. For instance, miR-106a-5p induces astrocytoma cell apoptosis mainly by targeting Fas-activated serine/threonine kinase (FASTK), and miR-429 enhances cell apoptosis in esophageal carcinoma mainly by suppressing the expression of Bcl-2 and SP-1 [26, 27]. Furthermore, miR-26b has been found to be upregulated in Alzheimer's Disease, and it significantly promotes post-mitotic neuronal cell apoptosis [28]. However, the role of miR-291b-3p in apoptosis has never been elucidated. MiR-291b-3p belongs to the murine miR-290 cluster, which includes miR-290-3p, miR-291a-3p, miR-291b-3p, miR-292-3p, miR-294 and miR-295 [29]. This cluster has been identified as a 2.2 kb region on chromosome 7, and it has been widely reported to regulate different cellular pathways by targeting various target genes [30]. For example, miR-291b-5p and miR-293 suppress embryonic stem cell pluripotency by suppressing the p65 subunit of NF- κ B [31].

We sought to elucidate the specific molecular mechanisms behind miR-291b-3p-induced NCTC1469 cell apoptosis. Using four popular miRNA target prediction methods, Miranda, TargetScan, PicTar and RNA22, we failed to identify any apoptosis-related proteins that could be potential targets of miR-291b-3p. However, we found that miR-291b-3p is highly conserved with miR-519b-3p, miR-519c-3p, and miR-519a. It is known that miR-519 directly regulates the expression of HuR, which has an mRNA sequence that is highly conserved between *Mus musculus* and *Homo sapiens*. HuR is mainly located in the nuclei of resting cells [32]. It has been reported that HuR can bind to ARE-containing mRNAs and be transported to the cytoplasm. The translocation of HuR from nucleus to cytoplasm is an important mechanism for stabilizing target mRNAs [33, 34]. Therefore, we aligned murine miR-291b-3p with the murine HuR mRNA and found that HuR may be a potential target

gene of miR-291b-3p. Direct binding of miR-291b-3p to HuR mRNA was evidenced by the luciferase assay: we found that overexpression of miR-291b-3p significantly impaired the luciferase activity in HEK 293T cells that were transfected with a luciferase reporter vector containing the HuR-CR region. Furthermore, luciferase activity was only slightly reduced in HEK cells that were co-transfected with the luciferase reporter vector containing the HuR-3'-UTR and the miR-291b-3p mimics, suggesting that miR-291b-3p negatively regulates HuR by direct binding to the coding region of HuR mRNA. These results were further validated by western blot, which showed that overexpression of miR-291b-3p reduced the protein level of HuR, while suppression of miR-291b-3p enhanced HuR expression. At present, the regulation of HuR by different miRNAs has been widely explored [35]. For instance, miR-122, miR-548c, miR-494, miR-16, and miR-331 have all been proven to target HuR in various cell types and tumor samples [18, 36-38].

The finding that HuR knockdown promoted NCTC1469 cell apoptosis confirmed the notion that HuR has an anti-apoptotic function. Previous research has already shown that reduced HuR expression confers a growth advantage in various malignant cells [39]. As one of the most important ARE-binding proteins, HuR binds to the ARE sequences in mRNAs with high affinity [40]. However, HuR exerts its effects in two distinctive ways. First, HuR can act as a positive regulator of gene expression by increasing the stability of target mRNAs, such as *cox-2*, *cyclin D1*, *p21* and so on [13, 41, 42]. However, HuR has also been found to decrease the translation efficiency of some mRNAs, such as *TNF α* , *c-myc*, and *p27* [43-45]. Recently, HuR expression level has been suggested to positively correlate with that of *Bcl-2* in HeLa cells [15, 46]. Abdelmohsen et al. indicated that HuR plays a positive role in regulating *Bcl-2* mRNA stability and translation efficiency [46]. Similarly, we have now shown that reducing the HuR level contributed to reduced expression of *Bcl-2*, which then prompted NCTC1469 cell apoptosis by increasing the *Bax/Bcl-2* ratio. Interestingly, we also noted higher *Bax* expression and lower *Bcl-2* expression when miR-291b-3p was upregulated. Both proteins belong to the *Bcl-2* family, but they have opposite functions during the apoptotic process [47]. Certainly, further investigation is needed to elucidate the mechanisms that lead to *Bax* upregulation during miR-291b-3p-induced apoptosis. We propose that miR-291b-3p may exert its function on *Bax* through other target genes than HuR. Nevertheless, our data clearly show that the *Bax/Bcl-2* ratio increased when HuR expression was blocked, resulting in cellular apoptosis.

In conclusion, we identified a novel pro-apoptotic role of miR-291b-3p through the modulation of HuR expression and the subsequent effect on *Bcl-2* stability in NCTC1469 cells.

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References

- 1 Milenkovic D, Deval C, Gouranton E, Landrier JF, Scalbert A, Morand C, Mazur A: Modulation of miRNA expression by dietary polyphenols in apoE deficient mice: a new mechanism of the action of polyphenols. *PLoS One* 2012;7:e29837.
- 2 Rottiers V, Naar AM: MicroRNAs in metabolism and metabolic disorders. *Nat Rev Mol Cell Biol* 2012;13:239-250.
- 3 Hu JZ, Huang JH, Zeng L, Wang G, Cao M, Lu HB: Anti-Apoptotic Effect of MicroRNA-21 after Contusion Spinal Cord Injury in Rats. *J Neurotrauma* 2013;30:1349-1360.

- 4 Farhana L, Dawson MI, Murshed F, Das JK, Rishi AK, Fontana JA: Upregulation of miR-150* and miR-630 induces apoptosis in pancreatic cancer cells by targeting IGF-1R. *PLoS One* 2013;8:e61015.
- 5 Sharma S, Verma S, Vasudevan M, Samanta S, Thakur JK, Kulshreshtha R: The interplay of HuR and miR-3134 in regulation of AU rich transcriptome. *RNA Biol* 2013;10: 1283-1290.
- 6 Wang J, Guo Y, Chu H, Guan Y, Bi J, Wang B: Multiple Functions of the RNA-Binding Protein HuR in Cancer Progression, Treatment Responses and Prognosis. *Int J Mol Sci* 2013;14:10015-10041.
- 7 Sahlberg AS, Ruuska M, Granfors K, Penttinen MA: Altered Regulation of ELAVL1/HuR in HLA-B27-Expressing U937 Monocytic Cells. *PLoS One* 2013;8:e70377.
- 8 Barreau C, Paillard L, Osborne HB: AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res* 2005;33:7138-150.
- 9 Peng SS, Chen CY, Xu N, Shyu AB: RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J* 1998;17:3461-3470.
- 10 Rodriguez-Pascual F, Hausding M, Ihrig-Biedert I, Furneaux H, Levy AP, Forstermann U, Kleinert H: Complex contribution of the 3'-untranslated region to the expressional regulation of the human inducible nitric-oxide synthase gene. Involvement of the RNA-binding protein HuR. *J Biol Chem* 2000;275:26040-26049.
- 11 Chen CY, Xu N, Shyu AB: Highly selective actions of HuR in antagonizing AU-rich element-mediated mRNA destabilization. *Mol Cell Biol* 2002;22:7268-7278.
- 12 Yaman I, Fernandez J, Sarkar B, Schneider RJ, Snider MD, Nagy LE, Hatzoglou M: Nutritional control of mRNA stability is mediated by a conserved AU-rich element that binds the cytoplasmic shuttling protein HuR. *J Biol Chem* 2002;277:41539-41546.
- 13 Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, Holbrook N, Gorospe M: HuR regulates p21 mRNA stabilization by UV light. *Mol Cell Biol* 2000;20:760-769.
- 14 Zou T, Mazan-Mamczarz K, Rao JN, Liu L, Marasa BS, Zhang AH, Xiao L, Pullmann R, Gorospe M, Wang JY: Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. *J Biol Chem* 2006;281:19387-19394.
- 15 Ishimaru D, Ramalingam S, Sengupta TK, Bandyopadhyay S, Dellis S, Tholanikunnel BG, Fernandes DJ, Spicer EK: Regulation of Bcl-2 expression by HuR in HL60 leukemia cells and A431 carcinoma cells. *Mol Cancer Res* 2009;7:1354-1366.
- 16 Leucci E, Zriwil A, Gregersen LH, Jensen KT, Obad S, Bellan C, Leoncini L, Kauppinen S, Lund AH: Inhibition of miR-9 de-represses HuR and DICER1 and impairs Hodgkin lymphoma tumour outgrowth in vivo. *Oncogene* 2012;31:5081-5089.
- 17 Kojima K, Fujita Y, Nozawa Y, Deguchi T, Ito M: MiR-34a attenuates paclitaxel-resistance of hormone-refractory prostate cancer PC3 cells through direct and indirect mechanisms. *Prostate* 2010;70:1501-1512.
- 18 Xu F, Zhang X, Lei Y, Liu X, Liu Z, Tong T, Wang W: Loss of repression of HuR translation by miR-16 may be responsible for the elevation of HuR in human breast carcinoma. *J Cell Biochem* 2010;111:727-734.
- 19 Al-Ahmadi W, Al-Ghamdi M, Al-Souhibani N, Khabar KS: miR-29a inhibition normalizes HuR over-expression and aberrant AU-rich mRNA stability in invasive cancer. *J Pathol* 2013;230:28-38.
- 20 Prislei S, Martinelli E, Mariani M, Raspaglio G, Sieber S, Ferrandina G, Shahabi S, Scambia G, Ferlini C: MiR-200c and HuR in ovarian cancer. *BMC Cancer* 2013;13:72.
- 21 Abdelmohsen K, Srikantan S, Kuwano Y, Gorospe M: miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels. *Proc Natl Acad Sci* 2008;105:20297-20302.
- 22 Dou L, Zhao T, Wang L, Huang X, Jiao J, Gao D, Zhang H, Shen T, Man Y, Wang S, Li J: miR-200s contribute to interleukin-6 (IL-6)-induced insulin resistance in hepatocytes. *J Biol Chem* 2013;288:22596-22606.
- 23 Abdelmohsen K, Kim MM, Srikantan S, Mercken EM, Brennan SE, Wilson GM, Cabo R, Gorospe M: miR-519 suppresses tumor growth by reducing HuR levels. *Cell Cycle* 2010;9:1354-1359.
- 24 Minero VG, Khadjavi A, Costelli P, Baccino FM, Bonelli G: JNK activation is required for TNFalpha-induced apoptosis in human hepatocarcinoma cells. *Int Immunopharmacol* 2013;17:92-98.
- 25 Shuh M, Bohorquez H, Loss GE, Jr, Cohen AJ: Tumor Necrosis Factor-alpha: Life and Death of Hepatocytes During Liver Ischemia/Reperfusion Injury. *Ochsner J* 2013;13:119-130.
- 26 Wang Y, Li M, Zang W, Ma Y, Wang N, Li P, Wang T, Zhao G: MiR-429 up-regulation induces apoptosis and suppresses invasion by targeting Bcl-2 and SP-1 in esophageal carcinoma. *Cell Oncol* 2013;36:385-394.

- 27 Zhi F, Zhou G, Shao N, Xia X, Shi Y, Wang Q, Zhang Y, Wang R, Xue L, Wang S, Wu S, Peng Y, Yang Y: miR-106a-5p Inhibits the Proliferation and Migration of Astrocytoma Cells and Promotes Apoptosis by Targeting FASTK. *PLoS One* 2013;8:e72390.
- 28 Absalon S, Kochanek DM, Raghavan V, Krichevsky AM: MiR-26b, Upregulated in Alzheimer's Disease, Activates Cell Cycle Entry, Tau-Phosphorylation, and Apoptosis in Postmitotic Neurons. *J Neurosci* 2013;33:14645-14659.
- 29 Marson A, Levine SS, Cole MF, Frampton GM, Brambrink T, Johnstone S, Guenther MG, Johnston WK, Wernig M, Newman J, Calabrese JM, Dennis LM, Volkert TL, Gupta S, Love J, Hannett N, Sharp PA, Bartel DP, Jaenisch R, Young RA: Connecting microRNA genes to the core transcriptional regulatory circuitry of embryonic stem cells. *Cell* 2008;134:521-533.
- 30 Zheng GX, Ravi A, Calabrese JM, Medeiros LA, Kirak O, Dennis LM, Jaenisch R, Burge CB, Sharp PA: A latent pro-survival function for the mir-290-295 cluster in mouse embryonic stem cells. *PLoS Genet* 2011;7:e1002054.
- 31 Luningschror P, Stocker B, Kaltschmidt B, Kaltschmidt C: miR-290 cluster modulates pluripotency by repressing canonical NF-kappaB signaling. *Stem Cells* 2012;30:655-664.
- 32 Yi X, Zhou Y, Zheng W, Chambers SK: HuR expression in the nucleus correlates with high histological grade and poor disease-free survival in ovarian cancer. *Aust N Z J Obstet Gynaecol* 2009;49:93-98.
- 33 Zhu Z, Wang B, Bi J, Zhang C, Guo Y, Chu H, Liang X, Zhong C, Wang J: Cytoplasmic HuR expression correlates with P-gp, HER-2 positivity, and poor outcome in breast cancer. *Tumour Biol* 2013;34:2299-2308.
- 34 Hostetter C, Licata LA, Witkiewicz A, Costantino CL, Yeo CJ, Brody JR, Keen JC: Cytoplasmic accumulation of the RNA binding protein HuR is central to tamoxifen resistance in estrogen receptor positive breast cancer cells. *Cancer Biol Ther* 2008;7:1496-1506.
- 35 Palanisamy V, Jakymiw A, Van Tubergen EA, D'Silva NJ, Kirkwood KL: Control of cytokine mRNA expression by RNA-binding proteins and microRNAs. *J Dent Res* 2012;91:651-658.
- 36 Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W: Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* 2006;125:1111-1124.
- 37 Tominaga K, Srikantan S, Lee EK, Subaran SS, Martindale JL, Abdelmohsen K, Gorospe M: Competitive regulation of nucleolin expression by HuR and miR-494. *Mol Cell Biol* 2011;31:4219-4231.
- 38 Epis MR, Barker A, Giles KM, Beveridge DJ, Leedman PJ: The RNA-binding protein HuR opposes the repression of ERBB-2 gene expression by microRNA miR-331-3p in prostate cancer cells. *J Biol Chem* 2011;286:41442-41454.
- 39 Dalmau J, Furneaux HM, Cordon-Cardo C, Posner JB: The expression of the Hu (paraneoplastic encephalomyelitis/sensory neuronopathy) antigen in human normal and tumor tissues. *Am J Pathol* 1992;141:881-886.
- 40 Al-Ahmadi W, Al-Ghamdi M, Al-Haj L, Al-Saif M, Khabar KS: Alternative polyadenylation variants of the RNA binding protein, HuR: abundance, role of AU-rich elements and auto-Regulation. *Nucleic Acids Res* 2009;37:3612-3624.
- 41 Kim GY, Lim SJ, Kim YW: Expression of HuR, COX-2, and survivin in lung cancers; cytoplasmic HuR stabilizes cyclooxygenase-2 in squamous cell carcinomas. *Mod Pathol* 2011;24:1336-1347.
- 42 Yuan Z, Sanders AJ, Ye L, Wang Y, Jiang WG: Knockdown of human antigen R reduces the growth and invasion of breast cancer cells in vitro and affects expression of cyclin D1 and MMP-9. *Oncol Rep* 2011;26:237-245.
- 43 Nabors LB, Suswam E, Huang Y, Yang X, Johnson MJ, King PH: Tumor necrosis factor alpha induces angiogenic factor up-regulation in malignant glioma cells: a role for RNA stabilization and HuR. *Cancer Res* 2003;63:4181-4187.
- 44 Talwar S, Jin J, Carroll B, Liu A, Gillespie MB, Palanisamy V: Caspase-mediated cleavage of RNA-binding protein HuR regulates c-Myc protein expression after hypoxic stress. *J Biol Chem* 2011;286:32333-32343.
- 45 Kullmann M, Gopfert U, Siewe B, Hengst L: ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5'UTR. *Genes Dev* 2002;16:3087-3099.
- 46 Abdelmohsen K, Lal A, Kim HH, Gorospe M: Posttranscriptional orchestration of an anti-apoptotic program by HuR. *Cell Cycle* 2007;6:1288-1292.
- 47 Hardwick JM, Soane L: Multiple functions of BCL-2 family proteins. *Cold Spring Harb Perspect Biol* 2013;5:pil: a008722. doi: 10.1101/cshperspect.a008722.