HuR Suppresses Fas Expression and Correlates with Patient Outcome in Liver Cancer

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List of Abbreviations:

HCC: hepatocellular carcinoma; HuR: human antigen R; FasL: Fas ligand; sFasL: super Fas ligand, UTR: untranslated region; BID: BH3 interacting-domain death agonist; PARP: poly(ADP-ribose) polymerase; PCR: polymerase chain reaction; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IgG: immunoglobulin G; SD: standard deviation; SEM: standard error of the mean

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

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ABSTRACT

Hepatocellular carcinomas (HCCs) show resistance to chemotherapy and have blunt response to apoptotic stimuli. HCC cell lines express low levels of the Fas death receptor and are resistant to FasL stimulation, whereas immortalized hepatocytes are sensitive. The variable Fas transcript levels and consistently low Fas protein in HCC cells suggests post-transcriptional regulation of Fas expression. The 3'-UTR of Fas mRNA was found to interact with the ribonucleoprotein Human Antigen R (HuR) to block mRNA translation. Silencing of HuR in HCC cells increased the levels of cell surface Fas and sensitized HCC cells to FasL. Two AU-rich domains within the 3'-UTR of Fas mRNA were identified as putative HuR binding sites and were found to mediate the translational regulation in reporter assay. Hydrodynamic transfection of HuR plasmid into mice induced downregulation of Fas expression in livers and established functional resistance to the killing effects of Fas agonist. Human HCC tumor tissues showed significantly higher overall and cytoplasmic HuR staining compared to normal liver tissues, and the high HuR staining score correlated with worse survival of patients with early stage HCC. Combined, the pro-tumorigenic ribonucleoprotein HuR blocks the translation of Fas mRNA and effectively prevents Fas-mediated apoptosis in HCC, suggesting that targeting HuR would sensitize cells to apoptotic stimuli and reverse tumorigenic properties.

Implications: Demonstrating how death receptor signaling pathways are altered during progression of hepatocellular carcinoma will enable the development of better methods to restore this potent apoptosis mechanism.
INTRODUCTION

HCC is the ninth leading cause of cancer-related deaths with a 5-year survival rate of 11%.(1-3) The immune surveillance system can identify cancerous cells and then eliminate them through Fas death receptor activation. However, tumor cells resist recognition by immune cells and apoptosis (4, 5). Healthy liver tissue expresses abundant levels of Fas that is typically silenced during HCC transformation, but HCC rarely acquires mutations that disable Fas signaling (4, 6).

HuR modulates post-transcriptional processing of target pre-mRNAs or mRNA stabilization and translation through interaction with AU-rich elements (AREs) within 3′-UTRs of the target mRNAs (7, 8). Loss of HuR leads to cell death and pathologic overexpression of HuR is almost uniformly linked to transformation (9). HuR has been previously shown to mediate skipping of Fas exon 6, leading to the production of a soluble decoy Fas (7, 10, 11). However, the relationship between HuR expression and modulation of Fas signaling in HCC has not been previously described.

In the current study, we revealed that elevated levels of HuR interfere with the translation of Fas mRNA, leading to decreased Fas expression and subsequent resistance to Fas-mediated apoptosis in HCC-derived cell lines and clinically aggressive HCC.
MATERIALS AND METHODS

Cell Culture
Terminally differentiated human hepatic cells HepaRG (EMD Millipore, Billerica, MA) were cultured in Williams E Medium with culture medium supplement (EMD Millipore). Immortalized normal hepatocytes (CRL4020) and HCC-derived cell lines (HepG2, Hep3B, SNU-182, SNU-398, SNU-449 and SNU-475) (ATCC, Manassas, VA) were cultured in Dulbecco’s modified Eagle medium. Myeloma cell line AMBL6 (a gift from Dr. Robert Orlowski, MD; The University to Texas Anderson Cancer Center) was maintained in RPMI 1640 medium. Both media were supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). All cell lines were regularly tested for mycoplasma (Lonza, Allendale, NJ).

Transient Knock-Down of HuR
Transient knock-down of HuR was achieved by electroporation of specific HuR-targeting SMARTpool-designed siRNAs (Sir-HuR) and the siCONTROL non-targeting siRNA (Sir-CON) (both from Dharmaco/Thermo Fisher Scientific, Pittsburgh, PA) into cells using the Neon Transfection System according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA) (12).

Cell Survival Assay
Cell survival was evaluated by the MTT assay according to the manufacturer’s protocol (Promega, Madison, WI) (12, 13).
Cell Death Assay

Treated cells were harvested, and incubated with propidium iodide (BD Pharmingen, San Diego, CA) before flow cytometry to measure the number of dead cells.

Immunoblot analysis

Cells and mouse livers were analyzed as described previously (12-14) using primary and secondary antibodies listed in the Supplement.

Confocal Microscopy

Fixed cells were incubated with anti-Fas (Abcam, Cambridge, MA) and anti-HuR (EMD Millipore) primary antibodies followed by Alexa Fluor 647-conjugated goat anti-mouse or Alexa Fluor 488-conjugated goat anti-rabbit secondary antibodies (both from Life Technologies). The stained cells were mounted in ProLong Gold antifade reagent with 4′, 6 diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies). Images of cells were acquired and analyzed as described previously (12).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated using the TRIzol reagent (Life Technologies) according to the manufacturer’s instructions. Complementary DNA was synthesized using a reverse transcription-PCR archive kit (Life Technologies) according to the manufacturer’s protocol. All qRT-PCR tests were performed using the ABI StepOnePlus Real-Time PCR-System (Applied Biosystems/Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Primer sequences and PCR amplification profile are listed in the Supplement.
Cloning

Two evolutionarily conserved AREs in the Fas 3′-UTR were cloned into a 3′-UTR-luciferase vector (OriGene, Rockville, MD) to make the constructs Luc-Seq1 and Luc-Seq2. The full-length Fas 3′-UTR luciferase vector was used as a template to amplify Seq1 and Seq2 using the primer pairs introducing AsiSI and XhoI restriction endonucleases sites (Listed in the Supplement). PCR products and vectors were digested with AsiSI and XhoI enzymes and ligated using T4 DNA ligase (all from New England Biolabs, Ipswich, MA).

Luciferase Reporter Assay

Lipofectamine 2000 (Life Technologies) was used to transfect HepG2 cells with plasmids: Luc-Fas 3′-UTR, Luc-Seq1 or Luc-Seq2, Sir-HuR or Sir-CON, Luc-Null, and Renilla luciferase reporter pRL-TK vector (both from Promega) as a transfection normalization control. Twenty-four hours after transfection, cells were harvested, and analyzed using dual-luciferase reporter assay kit (Promega). Mean luciferase activity levels were derived from 3 independent experiments, following normalization to the Renilla luciferase activity.

Ribonucleoprotein Immunoprecipitation

The ribonucleoprotein immunoprecipitation was performed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit for HuR (EMD Millipore) following the manufacturer’s instructions. Quantitative RT-PCR was conducted to determine the presence and quantities of Fas mRNAs.
**mRNA Stability Assay**

Cells were treated with actinomycin D (Sigma-Aldrich) for 8 or 24 hours. The total RNA was isolated and qRT-PCR was conducted as described above to determine the amount of Fas mRNA.

**Analysis of Newly Synthesized Proteins**

Cells were incubated in medium devoid of methionine (Promega) overnight prior to incubation with methionine mimic, Click-iT (L-azidohomoalanine) for nascent protein synthesis (Life Technologies), for 4 hours. Cells were then lysed for immunoblot analysis.

**Surface Fas and FasL Binding Assay**

Cells were collected and incubated with mouse IgG blocking reagent (Life Technologies) prior to incubation with the phycoerythrin-conjugated anti-Fas antibody UB2 (Beckman Coulter, Brea, CA) or the corresponding isotype control IgG1-phycoerythrin (BD Biosciences, San Jose, CA). To detect FasL binding, cells were incubated with FasL-FLAG (Enzo Life Sciences, Farmingdale, NY) followed by PhycoLink anti-FLAG-RPE antibody (ProZyme, Hayward, CA). Phycolink anti-FLAG-RPE staining without FasL-FLAG was used as negative control. Surface Fas and bound FasL were measured by flow cytometry and analyzed as described previously (12).

**Animal Experiments**

All animal experiments were performed in accordance with the guidelines of MD Anderson Cancer Center’s Institutional Animal Care and Use Committee. Five-to-six-week-old C57BL/6 mice (Harlan Laboratories, Indianapolis, IN) were hydrodynamically transfected with pcDNA3.1-HuR (a gift from Dr. Terry Dixon; Medical University of South Carolina) or pcDNA3.1 (14). Mice were challenged by an intraperitoneal injection of a lethal dose of the
agonistic anti-Fas antibody Jo2 (BD Biosciences) or buffer 24 hours after the transfection. Mouse survival was monitored for up to 12 hours after the challenge. Livers were excised at 12 hours or at the time of death for further analysis.

**Immunohistochemistry**

Tissue microarray slides of human HCC and normal liver tissues (IMH-360; IMGENEX/Novus Biologicals, Littleton, CO) were subjected to deparaffinization, rehydration and antigen retrieval prior to blocking using the BLOXALL kit (Vector Laboratories, Burlingame, CA) (15). Slides were then incubated with primary anti-HuR antibody (EMD Millipore) followed by biotinylated secondary antibody. The signal was enhanced with a VECTASTAIN Elite ABC Kit, and developed with a 3, 3’-diaminobenzidine (DAB) peroxidase kit (both from Vector Laboratories). Slides were counterstained using hematoxylin and mounted with Permount. HuR staining intensity and a percentage of positive cells were scored independently by two investigators using the following grading system: staining intensity (0, undetectable; 1, low; 2, moderate; and 3, high); percentage of positive cells (0, 0-24%; 1, 25-49%; 2, 50-74%; and 3, 75-100%) and cytoplasmic staining (0, absent; and 1, present). A HuR staining score was obtained from the sum of staining intensity and percentage of positive cell grades (0, 0-1; 1, 2-3; 2, 4; and 3, 5-6).

**Statistical Analysis**

Data are reported as the means ± standard deviation (SD) of three samples or the mean ± standard error of the mean (SEM) from three independent experiments unless indicated otherwise. Differences between groups were compared using the two-tailed Student’s t-test. Summary statistics including median and range for continuous variable age, frequency counts and percentages for categorical variables (such as stage and score variables) are reported. Fisher’s exact test was used to evaluate the association between score variables and stage. Wilcoxon rank sum test or Kruskal-Wallis test.
were used to evaluate the difference in age between/among patient groups. Kaplan-Meier method was used to estimate overall survival (OS). Median OS time in months with 95% confidence interval was calculated. The log-rank test was used to evaluate the difference in OS between patient groups. Statistical software SAS 9.1.3 (SAS, Cary, NC) and S-Plus 8.0 (TIBCO Software Inc., Palo Alto, CA) were used for all the analyses. $P$-value $< 0.05$ was considered statistically significant.

RESULTS

HCC Cell Lines Are Resistant to FasL.

Neither FasL nor sFasL suppressed the viability of HCC cell lines at the doses tested, whereas the highest doses significantly decreased the survival rate of HepaRG and CRL4020 cells (Fig. 1A). To explore the mechanism underlying the resistance of HCC cells to FasL, we evaluated the expression of Fas protein, Fas mRNA and Fas signaling-related molecules (FADD, pro-caspase 8, and BID). Fas protein levels were low to undetectable in all six HCC cell lines, but robust in HepaRG and CRL4020 cells (Fig. 1B) while the levels of Fas mRNA varied greatly among the HCC cell lines, but Fas mRNA was present in all cell lines except Hep3B (Fig. 1C). The expression levels of Fas signaling components were similar in all HCC cell lines to those found in CRL4020 cells with the exception of Hep3B cells that had undetectable levels of FADD, pro-caspase 8 and BID (data not shown). These results suggested that reduced levels of Fas in HCC cell lines are associated with their resistance to Fas-mediated apoptosis.
Expression of HuR Inversely Correlates with Expression of Fas in HCC Cell Lines.

Regulation of Fas isoform expression was shown to originate from HuR-promoted skipping of exon 6, leading to expression of soluble Fas instead of signaling-capable membrane-bound Fas receptor (7, 10). To determine whether Fas levels correlate with levels of HuR, we performed immunoblot analysis of HCC cell lines and control CRL4020 cells. All HCC cell lines showed higher levels of HuR compared CRL4020 cells (Fig. 2A). Confocal microscopy confirmed very low to undetectable levels Fas in HCC cell lines showing high HuR staining that extended into cytoplasm (Fig. 2B). The observed correlation between elevated expression of HuR and lack of Fas expression suggested that HuR might regulate Fas expression in HCC cell lines.

To assess HuR-promoted exon 6 skipping in our cell model, Fas pre-mRNA splicing was evaluated in HCC cell lines and CRL4020 cells by RT-PCR using primers flanking exon 6 of Fas. The significant amounts of a shorter band, corresponding to soluble Fas mRNAs, were generated from the positive control AMBL6 cells and Hep3B cells, while amounts of sFAS mRNA were comparable among the remaining HCC cell lines and control CRL4020 cells (Fig. S1A). These results suggested that alternative splicing of Fas pre-mRNA is not the primary mechanism of Fas downregulation in HCC cell lines.

HuR Binds to Fas mRNA.

The presence of HuR in the cytoplasm of HCC cell lines (Fig. 2B) suggested possible HuR involvement in regulation of Fas by exerting its effects on Fas mRNA. To confirm that cytoplasmic HuR operates normally in HCC cell lines, we evaluated previously reported effect of
HuR on FasL mRNA (16). As expected, HuR knock-down in HepG2 cells significantly lowered levels of FasL mRNA compared to control cells (Fig. S1B).

We thus shifted our focus to 3’-UTR of Fas mRNA that contains sequences with the potential to bind numerous regulatory proteins (http://rbpdb.ccbr.utoronto.ca/). Computational analysis of Fas mRNA using the UCSC Genome Browser (http://genome.ucsc.edu/) revealed 2 putative HuR binding sites (Seq1 and Seq2) in its 3’-UTR. A two-dimensional structure prediction algorithm (RNAfold, http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) further supported a high probability of HuR binding to these two conserved sites (Fig. 2C). Using a ribonucleoprotein immunoprecipitation followed by qRT-PCR we probed for a possible association between HuR protein and Fas mRNA in HepG2 cells. Fas mRNA was amplified from HuR-specific but not control IgG precipitates (Fig. 2D - left panel) that originated from samples with equivalent concentrations of input RNA containing comparable Fas mRNA levels (Fig. 2D - right panel). This newly identified association strongly implicated HuR in regulating Fas expression.

**HuR Regulates Fas mRNA Translation in HCC Cells.**

HuR typically stabilizes the transcripts bearing AREs in their 3’-UTRs or alters rates of translation of its mRNA targets (8, 9, 17). To reveal how HuR affects the Fas expression at the mRNA level, we selected readily transfectable HepG2 cells for further studies. We first knocked-down HuR in HepG2 cells using HuR-specific si-RNA (Sir-HuR) or control si-RNA (Sir-CON) (Fig. 3A). Quantitative RT-PCR showed no significant difference in the levels of Fas mRNA between HuR knock-down and control HepG2 cells (Fig. 3B). To check the stability of Fas mRNA, we blocked transcription in HuR-knock-down and control HepG2 cells and analyzed the levels of Fas mRNA over 24 hours. HuR knock-down and control cells showed no significant
differences in the rate of decay of Fas mRNA (Fig. 3C). We next examined the effect of HuR on Fas translation by using labeled amino acid incorporation. As shown in Fig. 3D, robust levels of Fas protein were produced in HuR-knock-down HepG2 cells, whereas little Fas was produced in control cells (Fig. 3D).

We next cloned the two putative HuR-binding sites (Fig. 2C) downstream of the luciferase reporter gene. HepG2 cells were transfected with Sir-HuR or Sir-CON combined with plasmids encoding luciferase reporter with the entire Fas mRNA 3′-UTR, Seq1 or Seq2, illustrated in Figure 3E. The reporter expression was completely blocked with Fas 3′-UTR, but only partially with Seq1 and Seq2 alone (Fig. 3E). HuR knock-down partially restored expression of the reporter with the entire Fas 3′-UTR, and fully restored expression of reporter with Seq1 and Seq2 (Fig. 3E). Thus, Seq1 and Seq2 blocked reporter expression in the presence of HuR. Taken together, these results suggested that HuR negatively affects translation of Fas rather than the stability of Fas mRNA and that this effect is at least partially mediated through HuR interaction with Seq1 and Seq2 in the 3′-UTR of Fas mRNA.

Knock-Down of HuR Increases Fas Expression, Binding of FasL and FasL-Induced Apoptosis.

To evaluate a more generalized effect of HuR on Fas protein expression, HuR was knocked down in three HCC lines (HepG2, SNU449, and SNU398) showing a wide range of Fas mRNA levels (Figure 1C). Silencing of HuR dramatically increased total Fas protein expression in all 3 cell lines (Fig. 4A) suggesting a generalized regulation of Fas expression by HuR in HCC cell lines.
Increased total cellular Fas protein expression in HuR knock-down HepG2 cells was accompanied by elevated surface Fas levels and subsequently also increased FasL binding, both detected by flow cytometry (Fig. 4B-C). Finally, sFasL induced 70.5 ± 1.7% cell death in HuR knock-down HepG2 cells, but only 22.8 ± 0.7% cell death in control HepG2 cells (Fig. 4D and Fig. S2A). Immunoblot analysis of cells stimulated by sFasL showed greater cleavage/activation of caspases -8 and -3 and lower BID levels in HuR knock-down than in control HepG2 cells (Fig. S2B), confirming that HuR-dependent regulation of Fas expression restores early Fas signaling and leads to an improved cell death response.

**Overexpression of HuR in Livers Lowers Fas Levels and Protects the Mice from a Lethal Challenge with Fas Agonistic Antibody.**

To assess the *in vivo* effects of HuR on Fas-mediated liver cell killing, we overexpressed HuR in mouse livers by hydrodynamic transfection and 24 hours later treated the mice with a lethal dose of Fas agonistic antibody Jo2 (12). HuR-transfected mice demonstrated significantly improved survival than did control vector-transfected mice (10 mice per group; \( P = 0.02 \)) (Fig. 5A). Gross examination of livers exposed to Jo2 revealed blackened liver tissue typical of extensive hemorrhaging in vector-transfected livers, but only limited blackening in HuR-transfected livers (Fig. 5B). Immunoblot analysis of liver tissues confirmed the overexpression of HuR, that was associated with lower levels of Fas and the apparently reduced processing of Fas signaling molecules (procaspses-3, -8 and , BID, and PARP) in HuR- compared to control vector-transfected mice after a challenge with Jo2 agonistic antibody (Fig. 5C). These *in vivo* data confirmed the cell culture dynamics showing that HuR blocks Fas-mediated apoptosis by
suppressing Fas protein expression and consequently decreasing the amplitude of Fas signaling that translates to lower rates of apoptosis.

**HuR is Overexpressed in HCC tissues.**

Consistent HuR overexpression in HCC cell lines compelled us to examine whether human HCC tumors also show elevated HuR expression. A liver cancer tissue microarray (TMA of 59 liver tissue cores from 44 patients; available characteristics are summarized in Table 1) was stained with anti-HuR antibody (Fig. 6A). Staining intensity, percentage of positive cells and cytoplasmic staining were evaluated independently by two researchers. HuR staining score was obtained by combining scores for the intensity and percentage of positive cells.

Our first objective was to evaluate the association between HuR staining score and disease stage for 55 tissue samples (4 cholangiocarcinoma tissues were excluded from the analysis). The association between HuR staining score (0-1 or 2-3) and disease stage (0, early [I/II], or late [III/IV]) was statistically significant ($P = 0.0013$) and showed a significant increasing trend (Chi-square for trend $P = 0.0007$) (Fig. 6B). Associations of scores for staining intensity, percentage of positive cells, and cytoplasmic staining with disease stage were also significant ($P$-values = 0.002, 0.002 and 0.02, respectively).

The second objective was to look at a possible association of disease stage and HuR staining score with the overall survival for 42 patients with HCC. We confirmed that patients with early stage (I/II) disease had better survival than late stage (III/IV) patients although differences showed marginal significance; $P$-value = 0.0579 (Figure 6C). There were no statistically significant differences in survival by gender ($P$-value = 0.1303) although, association between
stage of the disease (I/II and III/IV) and gender was statistically significant ($P$-value = 0.0471); 95% of stage III/IV disease patients were male compared to 68.2% of patients with stage I/II disease. Patients with the highest HuR staining score (score 3) had worse survival than patients with undetectable to moderate HuR staining score staining score (scores 0-2) although the observed differences were not statistically significant (Fig. 6D). Interestingly, analysis of survival of 22 HCC patients with the early stage (I/II) disease suggested that HuR staining score of 3 is associated with worse prognosis (Fig. 6E). However, the difference in OS between the undetectable to moderate (0-2) and high score groups (3) was not significant ($P$-value = 0.086). The 5-year OS rates for these groups were 0.78 (95% CI: 0.55, 1.00) and 0.38 (0.19, 0.76), respectively.

DISCUSSION

In this report, we show that HuR is consistently overexpressed in HCC-derived cell lines and HCC tumor tissues. We show an inverse correlation between the expression of HuR and the death receptor Fas in HCC-derived cell lines. HuR interacts with the 3′-UTR of Fas mRNA via 2 AREs to suppress its translation that explains the differential Fas expression in normal hepatocytes compared to HCC. We confirmed HuR-mediated Fas regulation in 3 HCC cell lines, normal liver cells and in mouse liver. In a cohort of unselected patients, the HuR levels correlated significantly with the advanced clinical HCC stage and high HuR staining was associated with shorter survival of patients with the early stage (stage I or II) HCC. The significance of the observed correlations is likely to be improved if larger cohorts of HCC patients were studied.
The properties of HuR protein extending beyond Fas regulation have generated wide interest. HuR controls the expression of a panel of proteins that are critical for transformation and aggressive tumor growth (9). Abundant expression of HuR correlates with aggressive growth of renal cell carcinoma, breast cancer, gastric cancer and a suggested correlation may hold true for HCC, as well (9, 18).

Under unstimulated conditions in nontransformed cells, HuR is expressed constitutively at low levels and primarily resides in the nucleus. Transformed cells or cells under stress show enhanced expression of HuR protein and enhance export of HuR mRNA to the cytoplasm. Several recent reports elucidated some of the mechanisms of HuR upregulation in HCC; hepatitis B encoded X protein (HBx) induces expression of HuR while murine double minute 2 (Mdm2) stabilizes HuR protein by NEDDylation and regulates its nucleocytoplasmic shuttling (19, 20). The cytoplasmic translocation is a prerequisite for HuR’s ability to regulate mRNAs stability or translation through binding to their AREs (15). HuR positively regulates the stability of over 80 mRNAs that encode proteins involved in apoptosis inhibition, inflammation and cell proliferation (9), including HAUSP, a regulator of p53 stability, during progression of nonalcoholic steatohepatitis to HCC (21). In contrast, the expression of a few HuR targets (c-myc, wnt5a, and p27) has been shown to be suppressed by HuR through blocking their translation (9, 17, 22, 23). In the case of the Fas receptor, HuR has been previously shown to mediate splicing out Fas exon 6, leading to the production of a soluble decoy Fas (7, 10, 11). Our data indicate that in HCC, HuR suppresses expression of the Fas receptor by blocking Fas mRNA translation without significantly affecting Fas mRNA stability or splicing. To our knowledge, this is the first report of HuR-mediated inhibition of Fas translation and confirms that
constitutive overexpression of HuR in HCC may be responsible for the low Fas expressing phenotype.

HuR blocks apoptosis and promotes survival by regulating mRNAs that encode proteins such as p21, Mcl-1, and Bcl-2 (24, 25). Our data revealed that in addition to the above mentioned apoptosis regulating proteins, HuR also blocks Fas-mediated apoptosis by interfering with the expression of Fas receptor. Using flow cytometry, we confirmed increased cell surface expression of Fas receptor and subsequently increased binding of FasL and apoptosis in HuR knock-down cells. Consistent with the cell and tissue analysis, the overexpression of HuR in mouse livers protected liver tissue from the injury induced by a Fas agonistic antibody, as evidenced by decreased liver hemorrhage, and attenuated apoptotic signaling in HuR-transfected mice compared with control vector-transfected mice.

The downregulation of Fas, gain of FasL, and production of soluble Fas are potential mechanisms contributing to HCC development (26, 27) and were implicated to occur as a coordinated event in HCCs. The existence of a common upstream regulator of Fas and FasL expression was proposed (28). Our findings indicate that HuR downregulates Fas and promotes expression of FasL in HCC suggesting that HuR is this sought common regulator.

HuR has been shown to suppress immunity through modulation of cytokines levels via regulation of their mRNAs. For example, HuR binds the mRNAs of MKP-1 and TGF-β and promotes their expression and coordinately suppresses tumor targeting immune responses (29, 30). HuR has a wider role in circumventing inflammation overall by post-transcriptional suppression of inflammatory cytokines production (17).
Alteration of the Fas/FasL system is regarded as a key component of immune evasion in HCC. One of the mechanisms of immune evasion is of HuR-mediated stabilization of FasL mRNA (16), which we confirmed also occurs in HCC cell lines as silencing of HuR decreased FasL mRNA levels. Our finding of HuR-mediated regulation of Fas expression fits well with the HuR functions in apparent evasion of immune surveillance (5). These events coupled with the wide ranging effects of HuR on apoptosis can shield tumors from the immune system.

Treatment of early stage HCC is by surgical resection. Use of targeted therapy with sorafenib and other chemotherapies shows incremental clinical improvements. However, most patients with non-resectable, late stage, HCC die within 3 to 6 months, and their 5-year survival remains a dismal 11% (1). Most effector responses to chemotherapy depend on an intact and inducible Fas death receptor signaling, which is typically blocked in HCC. Our analysis showed that interventions aimed to block HuR expression allow replenishment of Fas levels and confer cells with the capacity to respond to apoptotic stimulation with FasL. Restoration of Fas expression in HCC would enable effective apoptosis in response to chemotherapy treatments inducing cell killing. In other cancer settings, the direct modulation of Fas associated proteins (PMLRAR, nucleolin) determines the fate of cell apoptotic stimulus (12, 14).

Expression patterns of Fas and FasL have been used to distinguish cohorts of HCC patients with short (11 months) and long (52 months) disease-free intervals (27). Having recognized in our study that HuR regulates the expression of both Fas receptor and FasL, we anticipated that HuR expression levels would integrate the prognostic significance of Fas and FasL on survival of patients with HCC. Indeed, high expression of HuR was associated with shorter survival of patients with early stage HCC.
Having demonstrated a role of HuR in HCC apoptosis regulation, we queried whether HuR can be targeted pharmacologically. In order for HuR to exert its effects, it must dimerize prior to binding its targets. Low molecular weight inhibitors of HuR dimerization affected levels of HuR-targeted ARE-containing mRNAs and inhibited cell proliferation (31). Also, targeting of HuR-mRNA complexes by competing AREs has successfully displaced mRNAs leading to suppression of proliferation (32). Nuclear export of HuR is an almost-exclusive property of stressed or transformed cells and thus a promising target for therapy (33). Thus, targeting of HuR may offer us the ability to modulate a broad range of HuR-mediated pro-tumorigenic effects including cancer cell proliferation, survival, angiogenesis, invasion, and metastases by interfering with the actions of a single target (24, 25, 34-36).
FIGURE LEGENDS

**Fig 1.** Fas death receptor protein and mRNA levels and responsiveness of HCC cell lines to Fas activation. (A) Terminally differentiated human hepatic cells (HepaRG), human TERT immortalized hepatocytes (CRL4020), and six HCC cell lines were incubated with FasL or superFasL for 24 hours and cell survival was determined using MTT assay. ** P ≤ 0.01. (B) Cell extracts were analyzed by immunoblot analysis for basal expression of Fas relative to β-actin. (C) Total mRNA was isolated from the indicated cell lines and the content of Fas mRNA was evaluated relative to GAPDH mRNA by RT-PCR. Data in A and C are presented as the mean ± SEM of 3 independent experiments.

**Fig 2.** Expression of HuR in HCC cell lines and presence of putative HuR binding elements within the 3′-UTR of Fas. (A) Immunoblot analysis of Fas and HuR expression in HCC cell lines. Panels showing Fas and β-actin are the same as shown in Fig. 1B. (B) HepG2 cells stained by immunofluorescent anti-Fas (green) and anti-HuR (red) antibodies, and nuclear DAPI stain were observed by confocal microscopy. (C) Location and structure of 2 possible HuR binding sites within the Fas mRNA 3′-UTR. Dashed rectangles show location of conserved putative HuR binding sites Seq1 and Seq2. (D) Analysis of the HuR binding to Fas mRNA in HepG2 cells by ribonucleoprotein immunoprecipitation followed by qRT-PCR (left); analysis of input RNA (right).

**Fig 3.** The effect of HuR knock-down on the Fas mRNA levels, stability and translation in HepG2 cells. (A) Levels of HuR mRNA in HepG2 cells transfected with HuR-targeting si-RNA (Sir-HuR) or control si-RNA (Sir-CON) were evaluated by qRT-PCR. (B) The levels of Fas mRNA in Sir-HuR and Sir-CON transfected cells were analyzed by qRT-PCR. (C) Transcription was inhibited by incubation of cells transfected with Sir-HuR and Sir-CON with actinomycin D. Cells were harvested at indicated times post transfection and analyzed for expression of Fas mRNA by qRT-PCR. (D) Click-it protein synthesis assay was used to metabolically label Sir-HuR and Sir-CON transfected cells to evaluate translation of Fas mRNA by immunoblot analysis. (E) Schematic representation of luciferase reporter constructs with Fas 3′-UTR and identified HuR-binding sequences (Seq1 and Seq2), which were used to evaluate their effects on reporter translation in Sir-HuR or Sir-CON transfected cells by using dual-luciferase reporter assay. ** represent significant differences (P < 0.0001) between adjacent Sir-CON and Sir-HuR bars. Data in A–C are presented as the mean ± SEM of 3 independent experiments. Data in E represent mean ± SD of 6 separate wells. Experiment was repeated twice and the same trend was observed.
Fig 4. Effects of HuR knock-down on Fas protein expression, binding of FasL and apoptosis. (A) The expression of HuR and Fas detected by immunoblot analysis in indicated HCC cell lines transfected with Sir-HuR or Sir-CON. (B) Surface Fas was detected by flow cytometry of PE-conjugated Fas-antibody UB2-labeled Sir-HuR or Sir-CON transfected HepG2 cells. (C) The binding of FLAG-tagged Fas ligand to the surface of Sir-HuR or Sir-CON transfected HepG2 cells was evaluated by flow cytometry after staining with fluorescein-labeled anti-FLAG antibody. (D) Apoptosis of Sir-HuR or Sir-CON transfected HepG2 cells in response to FasL was evaluated by PI exclusion and flow cytometry. Graphical representation of the flow cytometry data in a bar graph is shown. Corresponding flow cytometry plots are provided in Fig. S2A.

Fig 5. Effect of HuR overexpression on mice survival and liver cell apoptosis after challenge with agonistic anti-Fas antibody. Mice were transfected with pcDNA3.1 or pcDNA3.1-HuR by hydrodynamic transfection method and 24 hours later they were challenged with a lethal dose of agonistic anti-Fas antibody Jo2. (A) Kaplan-Meier survival curves for pcDNA3.1 and pcDNA3.1-HuR transfected mice. (B) The gross appearance of livers from challenged and control mice. (C) Immunoblot analysis of Fas apoptotic signaling proteins in livers of transfected mice.

Fig 6. HuR expression and correlation with the stage of HCC and survival. Liver cancer TMA was stained with anti-HuR antibody and scored for staining intensity and percentage of positive cells independently by two investigators. An HuR staining score was obtained from the sum of scores for staining intensity and percentage of positive cells. (A) Examples of HuR staining in samples representing different stages of HCC. (B) Distribution of HuR staining score in stages of HCC. Increasing trend of HuR staining scores 2-3 with increasing HCC stage was significant; Chi-square for trend *P* = 0.0007. (C) Kaplan-Meier survival curves of 42 HCC patients with early (I/II) and late (III/IV) stage HCC. (*P* = 0.058). (D) Kaplan-Meier survival curves of 42 HCC patients with undetectable to moderate (0-1-2) and high (3) HuR staining scores disregarding the stage of HCC. (*P* = 0.264). (E) Kaplan-Meier survival curves of 22 HCC patients with early (I/II) stage HCC according to low (0-1-2) vs. high (3) HuR staining score. (*P* = 0.086).
REFERENCES


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Figure 1

A

Survival (% non-treated)

FasL (ng/mL)

FasL (ng/mL)

B

Fas mRNA Relative to HepaRG

C

Fas mRNA Relative to HepaRG

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Figure 2


(B) Immunofluorescence images demonstrating the localization of DAPI, Anti-Fas, and Anti-HuR in CRL4020 and SNU398 cell lines.

(C) Diagram of RNA secondary structure showing Seq1 and Seq2 regions.

(D) Bar graph illustrating the fold enrichment of Fas mRNA in IgG and anti-HuR conditions.
Figure 3

A  
HuR mRNA (%GAPDH)

Sir-CON  Sir-HuR

B  
Fas mRNA (%GAPDH)

Sir-CON  Sir-HuR

C  
Fas mRNA (%GAPDH)

Sir-CON  Sir-HuR

Time (hours)

D  

E  

Fas 3'UTR

Luc  Seq1  Seq2

Luc  Seq1

Luc  Seq2

Relative Luciferase Activity

Sir-CON  Sir-HuR

Luc-Null  Fas 3'UTR  Seq1  Seq2

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Figure 4

A

<table>
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<th>HepG2</th>
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<tr>
<td>SiRNA:</td>
<td>CON</td>
<td>HuR</td>
<td>CON</td>
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<tr>
<td>Fas</td>
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<td>HuR</td>
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<td></td>
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<tr>
<td>β-actin</td>
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</table>

B

- IgG
- Anti-Fas/Sir-CON
- Anti-Fas/Sir-HuR

C

- IgM
- Anti-FLAG/Sir-CON
- Anti-FLAG/Sir-HuR

D

Cell Death (% total)

- Sir-CON
- Sir-HuR

Non-treatment
sFasL (100ng/mL)

**
Figure 5

A

![Survival graph showing pcDNA3.1 and pcDNA3.1-HuR.](image)

B

![Images of tissues with and without Jo2.](image)

C

![Western blot analysis for HuR, Fas, Pro-caspase 8, Cleaved Caspase 8, Pro-caspase 3, Cleaved Caspase 3, BID, Cleaved PARP, and β-actin.](image)
Figure 6

<table>
<thead>
<tr>
<th></th>
<th>Normal Liver</th>
<th>Stage I</th>
<th>Stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
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<tr>
<td>Survival of stage I/II patients by HuR staining score</td>
<td>22.2%</td>
<td>77.3%</td>
<td>87.5%</td>
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<tr>
<td>Survival by stage</td>
<td>77.8%</td>
<td>22.7%</td>
<td>12.5%</td>
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</table>

Survival by stage:
- 1: I/II (E/N = 11/22)
- 2: III/IV (E/N = 14/20)

Overall Survival by Stage:
- 1: 0-1-2 (E/N = 7/15)
- 2: 3 (E/N = 18/27)

Overall Survival by NewScore:
- 1: 0-1-2 (E/N = 2/9)
- 2: 3 (E/N = 9/13)
Molecular Cancer Research

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Haifeng Zhu, Zuzana Berkova, Rohit Mathur, et al.

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