Signaling and Regulation

The RNA-Binding Protein HuR Promotes Glioma Growth and Treatment Resistance

Natalia Filippova¹, Xiuhua Yang¹, Yimin Wang³, G. Yancey Gillespie², Cathy Langford², Peter H. King¹,⁴, Crystal Wheeler¹, and L. Burt Nabors¹

Abstract

Posttranscriptional regulation is a critical control point for the expression of genes that promote or retard tumor growth. We previously found that the mRNA-binding protein, ELAV 1 (HuR), is upregulated in primary brain tumors and stabilizes growth factor mRNAs such as VEGF and IL-8. To better understand the role of HuR in brain tumor growth, we altered levels of HuR in glioma cells by short hairpin RNA or ectopic expression and measured tumor cell phenotype using in vitro and in vivo models. In HuR-silenced cells, we found a significant decrease in anchorage-independent growth and cell proliferation with a concomitant induction of apoptosis. Using an intracranial tumor model with primary glioblastoma cells, HuR silencing produced a significant decrease in tumor volume. In contrast, overexpression of HuR produced in vitro chemoresistance to standard glioma therapies. Because bcl-2 is abundantly expressed in glioma and associated with tumor growth and survival, we determined the impact of HuR on its regulation as a molecular validation to the cellular and animal studies. Using UV cross-linking and RNA immunoprecipitation, we show that HuR bound to the 3'-untranslated region of all bcl-2 family members. Silencing of HuR led to transcript destabilization and reduced protein expression. Polysome profiling indicated loss of HuR from the translational apparatus. In summary, these findings reveal a HuR-dependent mechanism for cancer cell survival and sensitivity to chemotherapeutic drugs suggesting that HuR should be considered as a new therapeutic target.

Mol Cancer Res; 9(5); 648–59. ©2011 AACR.

Introduction

Posttranscriptional regulation of RNA by RNA-binding proteins (RBP) and miRNA involves interactions with untranslated regions (UTR) of the mRNA, particularly the 3'-UTR (1–3). This level of gene regulation is essential for normal development but also is active in disease conditions such as cancer. The impact of RBPs and miRNAs on mRNA range from effects on stability, subcellular location, and/or efficiency of translation (4). Hu antigen R (HuR), is a member of the ELAV family that binds to adenine- and uridine-rich elements (ARE) located in the 3'-UTR (5). We have previously characterized expression patterns in cancers of the nervous system and identified several functional classes of targets important in glioma progression (6, 7). An expanding list of mRNAs regulated by HuR has been identified and include regulators of numerous cellular processes including inflammation (8, 9), cell cycle (10), angiogenesis (11), survival (12, 13), and apoptosis (14). Expression of HuR has also been characterized in cancers of the breast (15), ovaries (16), colon (17), and pancreas (18) and may be a biomarker for disease activity (19–24). In vivo studies indicate that elav1 knockout is embryonic lethal (25) and transgenic overexpression in specific tissue compartments alters the expression of ARE containing transcripts (26).

Glioblastoma (GBM) remains one of the most aggressive cancers with tremendous morbidity and mortality. The limited effectiveness of traditional cytotoxic therapies is most likely multifactorial; however, GBM is characterized by marked overexpression of the antiapoptotic bcl-2 family, which is associated with poor prognosis (27) and treatment resistance (28). In this work, we show that the conditioned expression of HuR with either inducible or silencing constructs alters cellular growth and sensitivity to apoptosis. Molecular analysis reveals that HuR binds to members of the bcl-2 family and promotes mRNA stability and protein expression. In addition, we show for the first time a

Authors’ Affiliations: Departments of ¹Neurology and ^Surgery, Division of Neurosurgery, University of Alabama at Birmingham; ^Division of Drug Discovery, Southern Research Institute, and ³Birmingham VA Medical Center, Birmingham, Alabama

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

N. Filippova and X. Yang contributed equally to this work.

Corresponding Author: L. Burt Nabors, Department of Neurology, University of Alabama at Birmingham, Neuro-oncology Program, 510 20th Street South, FOT 1020, Birmingham, AL 35294. Phone: 205-934-1432; Fax: 205-975-7546. E-mail: bnabors@uab.edu

doi: 10.1158/1541-7786.MCR-10-0325
©2011 American Association for Cancer Research.
significant impact of HuR on glioma tumor growth using an in vivo animal model. These observations support the viability of HuR as a novel molecular target in cancer.

Materials and Methods

Cell culture and expression of HuR
The U251 Tet-On cells were a gift from Dr. Erwin Van Meir (Emory University, Atlanta, GA). For stable transfections, pTRE2 plasmids were transfected into U251 Tet-On cells and the clones were selected with hygromycin. The maintenance, propagation, and transfection of U251 Tet-On cells are described elsewhere (7). Clones were selected with blasticidin and verified for transgene expression by Western blot using an anti-Flag antibody. The primary glioblastoma lines used for the in vivo experiments included the D456 glioma xenograft, a gift of Darell D. Bigner (Duke University, Durham, NC) and human lines GBM2, GBM10, and GBM12 were provided by David James and Jann Sarkaria (Mayo Clinic, Rochester, MN).

RNA interference
We used the SureSilencing shRNA (short hairpin RNA) plasmid (SABiosciences) for human ELAVL1 (UniGene# HS.184492) with the insert sequence 5'-GGAGGCTTCTCACTCATTGGAAGT-3' to generate stable clones. A random sequence 5'-GGAAATCTCTACATCGATCGATAC-3' was used for the control clones. Transfection of U251 was carried out with Mirus transfection reagent (Mirus Bio LLC) followed by selection with neomycin.

Western blotting, immunoprecipitation, and RNA immunoprecipitation
Cytosolic or whole cell lysates from cultured cells were prepared in the presence of protease inhibitors and sodium orthovanadate using the M-PER Kit (Pierce Endogen). One hundred micrograms of cell extract were incubated with 1 μL of antibody overnight at 4°C. The following antibodies were used: HuR (Santa Cruz Biotechnology) at 1:1,000, anti-HuC/HuD monoclonal 16A11 (Invitrogen), and FLAG (Sigma) 1:1,000 and equivalent amounts of control IgG (mouse IgG; Santa Cruz Biotechnology). One fifth of the supernatant served as a loading control. Protein G beads were added, and the antibody–antigen complex was then precipitated, washed, eluted in 1× Laemmli sample buffer, and subjected to SDS-PAGE electrophoresis followed by Western blot analysis. After immunoprecipitation (IP), RNA was eluted from protein G beads using the RNeasy Kit and analyzed by qRT-PCR mRNA. Standard real-time PCR amplification curves were generated (r² > 0.98) for bcl-2, mcl-1, and bcl-xl mRNA and 99 or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) controls using the threshold cycle (Ct) method. GAPDH primers and probe were obtained from Assays on Demand (Applied Biosystems). All qRT-PCR analyses were carried out on an ABI 7900 PCR instrument (Applied Biosystems). Western blot band density was determined with NIH ImageJ.

UV cross-linking
Nuclear extracts were prepared from U251 MG cells using the Nu-Per Kit (Pierce Endogen) and the protein concentration was determined with the BCA Protein Assay Kit. The UV cross-linking was carried out as previously described (7). The samples were electrophoresed on a 4% to 15% tris gradient gel (BioRad), dried and exposed on a phosphorimager. For immunoprecipitation, anti-HuR IgG or control IgG was added to the UV cross-linked sample in immunoprecipitation buffer as previously described (7).

Polyribosome isolation
Cells were grown to 80% to 90% confluency and treated for 15 minutes at 37°C and 5% CO2 with 100 mg/mL of cycloheximide in complete media (1× Dulbecco’s modified Eagle’s medium/F12K 50/50, 7% FBS). Cells were washed with PBS with cycloheximide (100 mg/mL) and trypsinized with 0.25% Trypsin/2.21 mmol/L EDTA and washed with ice-cold 1× PBS containing cycloheximide (100 mg/mL) and then pelleted. Protein collection, fractionation by sucrose gradient, RNA isolation, and analysis are as previously described (29).

Proliferation and soft agar assays
To measure cell proliferation, 5,000 cells were plated in 96-well plates and viable cell numbers were quantified using the ViaLight Kit (Lonza). Colony formation was carried out in SeaPlaque agarose (0.9%) with 8% fetal calf serum in 6-well plates. A cell density of 500 per well was used. After 4 weeks, 250 μL of p-iodonitroterazolium violet solution was added per well and incubated overnight. The following day, the plate was photographed and colony number quantified with NIH ImageJ software.

Chemosensitivity assays
The chemosensitivity assays were carried out by plating cells into 96-well plates. Before plating, cells were incubated for 24 hours in media containing doxycycline at 0.5 μg/mL for transgene induction. Chemotherapeutic agents were added to fresh culture media 24 hours after plating into 96-well plates. Cell viability was determined after 48 hours with the ViaLight Cytotoxicity assay. Three independent assays were carried out each in triplicate. For each construct, 2 independent clones were evaluated and averaged for each experiment. Data were analyzed with Prism software and fit to a nonlinear regression equation to describe the relationship between the log of the chemotheraphy concentration and the response (viable tumor cells): Y = 100/(1 + 10(log IC50/HuRslope)).
Generation of shHuR lentivirus
To generate the pLVTHM-shHuR vector shHuR primers were annealed and cloned into the MluI and ClaI sites of the pLVTHM vector. The control pLVTHM plasmid was obtained from Addgene, plasmid #12247. Viral particles were packaged by as previously described (29). A total of 10^6 U251 cells were infected with 2 mL of viral supernatant in the final volume of 4 mL. At 48-hour postinfection, cells were washed with DMEM-F12 media. Protein extracts and RNA from control and shHuR cells were obtained and analyzed after 4 weeks of cells culture expansion.

In vivo animal studies
All animal studies were carried out in accordance with the policies set by the UAB IACUC. Each mouse was placed in a stereotactic head holder with coordinates set to target the subcortical right frontal region. A linear incision (∼5 mm) was made in the scalp. A dental drill with a 0.45-mm noncutting bit was used to make a small burr hole and expose the dura. Using a 250-μL Hamilton syringe fitted with a 30-gauge needle, 0.5 × 10^6 or 10^6 GBM cells were injected. The burr hole was then filled with bone wax and the incision closed with glue.

Tumor analysis
Following euthanasia, the animals were decapitated. The brains were fixed in 10% zinc formalin overnight and stored. Coronal 30- to 50-μm brain slices (from front to back) were prepared using a Microm HM 355S (Alabama Neuroscience Blueprint Core C) and stored in antifreeze solution. The fluorescence spectrum from slices was obtained and analyzed on a model N-msi-500-FL multispectral imaging system (Leica Microsystems, Wetzlar GmbH, type 307-072.55), GFP filter, and Nuance software (Cambridge Research Instrumentation) in the UAB Small Animals Imaging Facility. To quantify tumor size in each brain section, a background coronal image was subtracted from a fluorescent image leaving the tumor whose area was calculated by using the NIH Imagej program. Tumor size was determined on every sixth 40-μm slice for each brain. Nineteen slices (from olfactory bulb through cerebellum) were analyzed for each animal, and tumor size was estimated by totaling the tumor area for all sections.

Statistics
A paired Student’s t test was applied for statistical analysis using GraphPad Prism v.4.

Results
HuR is required for anchorage-independent growth, survival, and chemoresistance
We investigated the role of HuR in promoting the transformed phenotype of glioma cells. Cells from shControl or shHuR were plated and assessed for proliferation over 4 days. We observed a 50% reduction in cell number, as determined by ATP bioluminescence, in shHuR cells relative to control (Fig. 1A). This difference was significant (P = 0.03). We next assessed colony formation in soft agar. Colonies formed by shHuR clones (n = 2) were strikingly sparse and small compared with shControl (n = 2; Fig. 1C, left, bottom vs. top row). Colony counts from 3 independent experiments showed a significant 10-fold reduction of colonies in shHuR clones compared with control (P = 0.0016). Together, these data support a role for HuR in proliferation and growth of malignant glioma.

To determine if growth inhibitory effects of HuR knockdown were due, in part, to the induction of apoptosis or cell-cycle arrest, we examined regulators of apoptosis (Fig. 1B). Knockdown resulted in a time-dependent appearance of cleaved caspase-3 suggesting that HuR silencing induced cell death through a caspase-3-dependent process. The presence of cleaved PARP further supports activation of the caspase cascade. Analysis of cell-cycle distributions in control and HuR silenced clones did not reveal a difference (data not shown). These results suggest that apoptotic cell death, induced by loss of HuR, contributes to growth inhibition. The effect of standard chemotherapy on glioma viability was analyzed in the setting of HuR knockdown. Inhibitory concentration 50 (IC_{50}) curves were generated for shHuR, shControl, and wild-type U251 cells (Fig. 1D). Compared with control cells, there was a 2- to 3-fold reduction of IC_{50} values for all the 3 agents. Wild-type U251 had similar IC_{50} values as shControls (data not shown).

The ability of HuR to influence glioma chemoresistance was evaluated by examining HuR overexpression. We used U251 clones that over express Flag-tagged HuR following doxycycline induction (7). To determine whether ectopically expressed Flag-HuR increased overall binding to bcl-2 mRNA, we carried out RNA immunoprecipitation (RIP) in Flag-HuR cells following doxycycline induction (Fig. 2A). We quantified the amount of bcl-2 mRNA precipitated and expressed it as fold increase over noninduced cells. With HuR induction, we found a significant increase in bcl-2 mRNA recovered by IP in the induced clones (140-fold for bcl-2, 64-fold for mcl-1, 40-fold for bcl-XL), but not GAPDH. The RIP assay also confirms the functionality of the induced Flag-tagged HuR. We treated the cells with etoposide, cisplatin, and topotecan (Fig. 2B). In the bottom panel, the IC_{50} curves are shifted to the right following induction of Flag-HuR with doxycycline, indicating a resistance to chemotherapy cytotoxicity. Controls included the parent clone, U251 Teton, and uninduced Flag-HuR clones. The increase in IC_{50} values of 0.5 to 1 log difference is biologically and clinically significant. In summary, the cellular experiments indicate an important role for HuR in glioma proliferation and chemotherapeutic resistance.

HuR binds to the 3’-UTR of the Bcl-2 family and regulates expression
The bcl-2 family of antiapoptotic genes have large AREs in their 3’-UTRs that could serve as targets for HuR binding. An interaction between the bcl-2 family RNA and HuR would implicate the process of posttranscriptional regulation in the promotion of glioma growth and resistance.
to cytotoxic agents. To determine whether HuR interacts with bcl-2 3'-UTR in glioma cells, we carried out UV cross-linking of U251 glioma extract with radiolabeled 3'-UTR probes from all 3 members of the bcl-2 family (Fig. 3 and Supplementary Fig. S1). In Figure 3A, the pattern of the UV cross-linking is seen in lane 1 for each riboprobe. The multiple bands are similar across the various 3'-UTR riboprobes suggesting interactions with similar groups of proteins. To determine a specific interaction with HuR, we carried out an immunoprecipitation of the cross-linked extract with an anti-HuR antibody. We detected radiolabeled bands representative of HuR for all 3 bcl-2 family members (Fig. 3A, lanes 2). No bands were detected with IgG control (lanes 3). Using the mcl-1 3'-UTR, we mapped the binding to areas generally rich in AREs. The right panel of Figure 3A is a UV cross-linking experiment with 4 fragments of the mcl-1 3'-UTR. Following IP with HuR antibodies, the most intense bands were detected in fragments 2 and 3, which have the highest alanine uracil (AU) content. There was no interaction with the 3'-UTR.
riboprobe mcl-1 #1, which has the lowest AU content. A faint band may be present for mcl-1 #4, the distal most fragment of the 3'-UTR.

To quantify the interaction between HuR and the bcl-2 family, we immunoprecipitated HuR from U251 extracts, purified the bound mRNA, and quantified the mRNA targets. Bound target mRNA was expressed as a percentage of total target mRNA for the specific target in the supernatant (Fig. 3B). An isotype-specific mouse IgG was included as a control. The bar graph illustrates increased amounts of bound bcl-2 family members with the HuR antibody compared with control IgG. GAPDH does not have an ARE and showed no binding difference between HuR and control IgG. Taken together, these results support a direct interaction between HuR and the 3'-UTRs of the bcl-2 family.

The impact of HuR binding to bcl-2 mRNA was determined by HuR knockdown. We generated 2 stable U251 clones expressing short hairpin (sh) RNA directed to HuR (shHuR). A scrambled sequence was utilized to generate control (shControl) clones. Effective and specific knockdown of HuR was observed at the mRNA and protein levels (Supplementary Fig. S1). The shHuR clones showed an 80% reduction in HuR mRNA compared with shControl clones. Western blot confirmed a specific reduction in HuR protein without affecting the highly homologous ELAV family member, HuC.

The functional consequence of the HuR interaction with the bcl-2 families’ 3'-UTR was examined by determining the mRNA half-life (t1/2). The t1/2 of all members was reduced in shHuR cells by more than half that of control shRNA (from 4–6 hours to less than 2 hours).
The data represent an average of 2 independent clones for shControl and shHuR. Although there was a significant reduction in mRNA half-life for each member, the overall mRNA levels were not significantly reduced in shHuR cells (Fig. 4A). The change in RNA degradation had a significant impact on protein expression of bcl-2, mcl-1, and bcl-XL. Western blot analysis of protein extracts from shControl and shHuR clones is shown in Figure 5A. After immunoblotting for bcl-2, mcl-1, or bcl-XL, markedly diminished bands are present in the shHuR clones but normal levels are present in the shControls when compared with wild-type U251. We identified 2 independent HuR-silenced clones with significant reductions in all 3 members of the bcl-2 family (noted with asterisks) and were the ones used for phenotypic studies. Because HuR plays an integral role in mRNA localization to the polysome, we next examined the distribution of RNA in the translational machinery. We isolated polysome fractions by sucrose gradient and then measured mRNA targets in those fractions by qRT-PCR. The polyribosome profiles were assessed by UV spectrophotometry with fractions 1 to 2 representing non-ribosomal components, fractions 3 to 6 the ribosomal components (subunits 18 S and 28 S), and fraction 7 to 12 polyribosomes (Fig. 5B). We analyzed the fractions by Western blot and found a decrease in HuR from shHuR clones, particularly in the polyribosome fractions. The distribution of ribosomal protein S6 and eIF4E is provided as controls for the integrity of fractionation. The distribution profiles differ slightly for the bcl-2 family members with a general shift toward more RNA within the polyribosome fractions (7–12) in the clones with HuR silenced compared with the control clones (Fig. 5C). The pattern was similar to GAPDH RNA profile, a species which lacks an AU-rich 3′-UTR. These data support a functional role of HuR in the posttranscriptional regulation of the bcl-2 family, with the impact primarily on the mRNA destabilization and serve as a molecular validation of a HuR silencing effect.

In vivo effect of HuR silencing on primary GBM xenografts

To evaluate the effect of HuR protein knockdown on in vivo tumor growth, we cloned different shHuR sequences (V1–3) into the lentiviral vector pLVTHM (Fig. 6A). This vector also contains the coding sequence for green fluorescent protein (GFP) to monitor infectivity. Initial characterization of 3 shHuR lentiviral constructs (V1–3) was carried out in U251 cells (Fig. 6A) and showed robust HuR knockdown at the RNA and protein levels (Fig. 6B). Control lentivirus (V-GFP) had no effect on HuR expression. In conjunction with this knockdown, we observed a reduction in bcl-2 protein (Fig. 6B). For in vivo characterization of HuR knockdown, we chose primary human GBM cell lines passaged subcutaneously in mice to maintain a tumor phenotype more reflective of human disease than established glioma cell lines (30, 31). A total of 4 primary GBM
lines (D456, GBM12, GBM6, and GBM10) were infected with V-GFP or V2shHuR lentivirus illustrated in Figure 6C and D. HuR silencing in primary GBM lines infected with V2shHuR was 70% to 80% compared with V-GFP control. Figure 6E documents a Western blot for the primary GBM line, D456 with knockdown of HuR with V2shHuR.

Infected primary glioma cells were injected intracranially into the right frontal subcortex of mice. The in vivo experiments were completed on 3 sets of animals using the primary GBM lines D456, GBM6, and GBM12. Each set of 10 mice received a different primary GBM line with 5 animals receiving cells infected with the V2shHuR construct and 5 animals the control (V-GFP). Mice were sacrificed 3 to 4 weeks after tumor cell injection and whole brains were imaged for GFP fluorescence. Figures 7 and 8 and Table 1 represent results from the D456 primary GBM line. We observed a marked increase in tumor initiation and dissemination in the control V-GFP control animals compared with those with HuR knockdown (V2shHuR) suggesting a growth disadvantage for HuR-silenced tumors (Fig. 7A). There was significant attenuation of tumor growth and invasiveness in the shHuR tumors compared with control. Tumors were analyzed in detail on 40-μm-thick coronal brain sections (rostral to caudal) using a stereo fluorescence microscope for GFP detection (Fig. 7B). The control and shHuR mice groups for D456 and GBM12 had similar patterns of tumor distribution within the area of injection; however, control animals had tumor cell migration from right to the left hemispheres, dissemination into and through the lateral ventricles, the striatum, hippocampus, and the third ventricle. GBM6 produced multifocal disease in control mice (defined as tumor populations completely separated from the primary injection site and not in continuity with other areas of tumor). There was a greater tumor size in V-GFP control mice (shControl) compared with those with HuR knockdown (V2shHuR).
with V2shHuR mice for all 3 lines (D456, GBM6, and GBM12). In Table 1, we provide a table of the data as the absolute total tumor area in shControl and shHuR. There was a difference in tumor size development across the 3 cell lines with consistent trends in control animals (wild-type HuR) having larger tumors than HuR-silenced animals (shHuR). This variation may reflect difference in behavior of the primary GBM lines with some growing faster than others. The time from implantation to animal sacrifice was the same for all at 3 weeks. The difference was statistically significant for D456 and GBM6 and approached for GBM12. GBM12 had a greater SEM suggesting greater variability in tumor behavior in the model. This could be technical or biologically driven by this particular primary line. The distance of saggital (rostral/caudal) migration of tumor cells was greater for control group compared with shHuR group. Tumor cells were detected from olfactory bulb to cerebellum in 4 of 5 control mice compared with only 1 of 5 mice in the shHuR group for both D456 and GBM12. GBM6 had a very different phenotype compared with the others. It appeared to disseminate more through the brain with numerous tumor foci. The difference in the number of discrete tumor foci was statistically significant with few following HuR silencing compared with the control (shControl). The number of tumor foci were counted and reported in Table 1. For this primary line, the effect of HuR silencing appears to impact both growth and migration. Overall, the primary GBM xenografts displayed more variability than would be seen with established lines but were clearly rendered less proliferative and invasive following HuR silencing.

We next analyzed immunohistochemical features of the tumors. shControl tumors were more invasive in appearance compared with shHuR animals (Fig. 8A). Staining of tumor sections confirmed a reduction in HuR in the shHuR animals by both immunohistochemistry and immunofluorescence (Fig. 8B). The impact of HuR silencing on bcl-2 protein extended to the in vivo setting with a reduction in bcl-2 immunofluorescence in shHuR animals versus the shControls as shown in Supplementary Figure S2. Thus, our data suggest that HuR knockdown significantly decreases tumor proliferation and migration in an orthotopic in vivo mouse model with primary GBM xenograft lines.

**Figure 5.** Impact of HuR knockdown on bcl-2 family RNA distribution within the polyribosome and protein expression. A, the silencing of HuR in multiple clones with the concurrent reduction in protein expression of all bcl-2 family members in select clones (*) which were used for phenotypic studies. Band density was normalized to tubulin and then compared with U251 to generate percent expression (shown below the blot). B, Western blot analysis from protein samples of 12 fractions collected following polyribosome isolation. S6 and elf4E are ribosomal components and used for control. HuR is reduced in the polysome fractions of shHuR cells compared with controls. C, the quantitation of RNA from each fraction for GAPDH and the bcl-2 family. Overall, the patterns differ depending on the target with a slight increase in distribution to later fractions (8–12) following HuR knockdown. The data are presented so that each bar represents the percentage of RNA from that fraction divided by the total for all 12 fractions.
Aberrant regulation of gene expression in cancer is typically attributed to transcriptional control. The molecular events occurring after production of the mature mRNA molecule (posttranscriptional regulation); however, have a substantial impact on genes that fuel tumor growth (3). Our work illustrates this impact for the first time using...
an animal model of primary malignant glioma. We also expand the mRNA targets for HuR in cancer to include the \textit{bcl-2} family.

Expression of HuR in cancer was first described by our group in primary brain tumors but has been extensively expanded by others to include multiple tumor types (6). In normal growth and development, HuR is primarily located in the nucleus; however, in malignant tumors a cytoplasmic shift has been reported that correlates with a worse prognosis (20). Alterations in expression and sub-cellular localization of HuR suggest a role for posttranscriptional regulation in multiple cancer-associated pathways such as cell cycle, inflammation, invasion and metastasis, angiogenesis, survival, and therapeutic resistance. The number of potential HuR-regulated mRNAs active in these disease pathways is potentially large as up to 8\% of the transcribed genome may contain an AU-rich 3'-UTR (32). Here, we have focused on the \textit{bcl-2} family as a molecular target of HuR. While other mRNA targets may be affected by HuR and contribute to the tumor phenotype, the \textit{bcl-2} family is implicated as an important control point in glioblastoma survival and treatment resistance, and it serves as a molecular validation for our tumor models. Utilizing established glioma cell lines and primary GBM lines, we have extended previous reports and identified the entire \textit{bcl-2} family as a candidate for regulation by HuR (14). All family members (\textit{bcl-2}, \textit{mcl-1}, and \textit{bcl-xl}) contain a 3'-UTR rich in AREs which serve as binding targets for HuR. This interaction promotes expression of the protein product by reducing RNA degradation. In addition, we observed a loss of HuR in the polysomes following silencing with substantial decrease in \textit{bcl-2} family member RNA levels in the polysomes suggesting HuR may stimulate translation of this family as described by other for XIAP (X chromosome-linked inhibitor of apoptosis; ref. 33).

Our data support a role for HuR in cell survival more than proliferation. Thus, we saw a significant induction in apoptosis and loss of anchorage-dependent growth with HuR knockdown but no impact on cell-cycle distribution. Likewise, overexpression of HuR in glioma cell lines resulted in significantly greater resistance to cytotoxic chemotherapy agents (Fig. 2). These results suggest that inactivation of HuR, as a molecular target in glioma tumors, could provide therapeutic synergy with traditional chemotherapy. With knockdown of HuR, the enhanced cytotoxicity at lower concentrations would ameliorate 2 clinically relevant limitations for chemotherapy: systemic toxicities and poor penetration into the brain.

### Table 1. Data for the 3 sets of animal experiments with each primary GBM line

<table>
<thead>
<tr>
<th>Primary GBM line</th>
<th>shControl Mice</th>
<th>SEM</th>
<th>shHuR Mice</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary endpoint is tumor area mm$^2$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D456</td>
<td>98</td>
<td>5</td>
<td>62</td>
<td>5</td>
<td>0.0116</td>
</tr>
<tr>
<td>GBM12</td>
<td>31</td>
<td>5</td>
<td>12</td>
<td>5</td>
<td>0.10</td>
</tr>
<tr>
<td>GBM6</td>
<td>1.0</td>
<td>0.06</td>
<td>0.04</td>
<td>5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Primary endpoint is number of distinct tumor foci

| GBM6 | 41 | 9 | 2.4 | 5 | 0.0002 |

**NOTE:** When tumor size is the primary endpoint, a consistent size difference is seen following silencing of HuR (shHuR). GBM6 shows a very migratory behavior and a difference in tumor foci is quantified as a second primary endpoint.
The transition of molecular and cellular studies to animal models has been undergoing a rapid evolution in the field of glioma research and other cancers. The traditional intracranial model using established glioma cell lines does not recapitulate the pathological or clinical behavior of human disease. To address this deficiency, we used patient-derived primary GBM xenograft lines. This model shows rapid tumor progression with extensive central nervous system dissemination and mimics human disease (30, 31). By the use of a lentiviral vector, we were able to knockdown HuR in a panel of primary GBM lines before implantation. The resultant effect was 2-fold: (i) a significant diminution of tumor growth and (ii) significantly less invasion. The behavior of the patient-derived tumors did vary, as with human disease, with some more aggressive and highly invasive and others those were smaller but multifocal. With all cell lines, however, the tumors were smaller, less invasive, or more unifocal with HuR knockdown. These results support our hypothesis that HuR is important in glioma growth and the viability of this RNA-binding protein as a therapeutic target in malignant glioma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This study was supported by NCI R01 CA112397 (L.B. Nabors), VA Merit Review (P.H. King), and by UAB Small Animal Imaging Shared Facility (P30CA013148).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 21, 2010; revised March 11, 2011; accepted March 12, 2011; published OnlineFirst April 15, 2011.

Figure 8. HuR knockdown reduces GBM tumor growth in vivo. A, coronal sections of mouse brains injected with shControl or V2shHuR-infected D456 GBM cells are shown. V2shHuR cells produced a smaller and less infiltrative tumor compared with shControl (arrows). B, representative tumor sections immunostained for HuR (red) and counterstained with DAPI (blue) showing reduced expression of HuR in the V2shHuR-infected GBM cells.

References


The RNA-Binding Protein HuR Promotes Glioma Growth and Treatment Resistance

Natalia Filippova, Xiuhua Yang, Yimin Wang, et al.