The Oncogenic RNA-Binding Protein Musashi1 Is Regulated by HuR via mRNA Translation and Stability in Glioblastoma Cells


Abstract

Musashi1 (Msi1) is an evolutionarily conserved RNA-binding protein (RBP) that has profound implications in cellular processes such as stem cell maintenance, nervous system development, and tumorigenesis. Msi1 is highly expressed in many cancers, including glioblastoma, whereas in normal tissues, its expression is restricted to stem cells. Unfortunately, the factors that modulate Msi1 expression and trigger high levels in tumors are largely unknown. The Msi1 mRNA has a long 3′ untranslated region (UTR) containing several AU- and U-rich sequences. This type of sequence motif is often targeted by HuR, another important RBP known to be highly expressed in tumor tissue such as glioblastoma and to regulate a variety of cancer-related genes. In this report, we show an interaction between HuR and the Msi1 3′-UTR, resulting in a positive regulation of Msi1 expression. We show that HuR increased Msi1 mRNA stability and promoted its translation. We also present evidence that expression of HuR and Msi1 correlate positively in clinical glioblastoma samples. Finally, we show that inhibition of cell proliferation, increased apoptosis, and changes in cell-cycle profile as a result of silencing HuR are partially rescued when Msi1 is ectopically expressed. In summary, our results suggest that HuR is an important regulator of Msi1 in glioblastoma and that this regulation has important biological consequences during gliomagenesis. Mol Cancer Res; 10(1); 143–55. ©2012 AACR.

Introduction

Cancer is a disease process ultimately triggered by aberrant gene expression. In this scenario, there is a large body of literature discussing the participation of transcription and chromatin modification factors in tumorigenesis. On the other hand, the role of posttranscriptional regulation in tumor growth is still poorly comprehended. RNA-binding proteins (RBP) are key mediators of posttranscriptional gene regulation, mediating many aspects of RNA metabolism such as splicing, transport, localization, translational regulation, and stability (1). In addition to controlling normal processes in cellular physiology, aberrant expression of RBPs has been shown to lead to tumorigenesis when their target genes are implicated in cell proliferation, differentiation, apoptosis, invasion, and metastasis.

An RBP of particular interest in cancer is Musashi1 (Msi1), first described as a gene required for the development of the Drosophila adult external sensory organ (2). In mammals, Musashi1 is required for nervous system development during embryonic development, whereas in adults, Musashi1 expression is restricted to stem and progenitor cells of various tissues (3–6). Aberrantly high Musashi1 expression is observed in many cancers such as medulloblastoma (7), hepatocellular carcinoma (8), cervical adenocarcinoma (9), lung cancer (10), colon cancer (11), and glioblastoma multiforme (GBM). In fact, increasing expression of Musashi1 has been correlated with a poor prognosis in glioma (12), breast cancer (13), and medulloblastoma (Penalva Lab, unpublished data).

During normal development, Musashi1 maintains stem cell identity, serving as a key gene in stemness (14). However, in the tumor environment, Musashi1 enhances cancer features. High Msi1 expression is associated with increased Notch1 expression and areas of tumor invasion/metastasis (12). Notch is a critical pathway for tumorigenesis in medulloblastoma, glioblastoma, and other tumor types; Msi1 influences this pathway by repressing the regulation of NUMB mRNA, a negative regulator of Notch (15–17). In medulloblastoma, Msi1 inhibition results in increased sensitivity to the Hedgehog pathway inhibitor cycloppamine,

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doi: 10.1158/1541-7786.MCR-11-0208

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indicating that Msi1 interfaces with the Hedgehog pathway (18). In murine in vivo xenograft experiments using breast and colon cancer cells, silencing of Msi1 via siRNAs results in inhibition of tumor growth (13, 19); similar results were observed for glioblastoma and medulloblastoma cells (Penalva Lab, unpublished data). Our results indicate that Msi1 influences tumor progression in a complex manner by regulating the expression of a network of genes implicated in cancer-related processes such as cell proliferation, apoptosis, cell cycle, and differentiation (17).

As summarized above, a large amount of data support the role of Msi1 as an oncogenic protein. However, the molecular determinants of increased Musashi1 expression during tumorigenesis are largely unknown. In normal stem cells, one study identified the HuD RBP as a potential posttranscriptional regulator of Musashi1 expression, aiding neural stem cells in the transition toward differentiation (20). It has also been suggested that a potential regulatory element at the transcriptional level exist as evident by the presence of a hypoxia-responsive element which can bind the hypoxia-inducible factor 1 in times of hypoxic stress, promoting self-renewal and proliferation in neural stem cells (21). However, neither of these elements can explicate the overexpression of Musashi1 during tumorigenesis. The mRNA of Msi1 contains a long 3′ untranslated region (UTR), spanning approximately 1,800 nucleotides, making it a likely candidate for posttranscriptional regulation. We have recently shown that Msi1 expression is regulated by several tumor suppressor microRNAs (22). Furthermore, the 3′-UTR contains several segments of AU- or U-rich cis-regulatory sequences, making it a potential target of turnover and translation regulatory RBP (TTR-RBP) such as HuR (also known as ELAVL1 or HuA), a member of the Hu/ELAV (embryonic lethal abnormal vision) family (23).

HuR can enhance tumorigenesis by interacting with a subset of mRNAs that encode proteins that regulate cell proliferation, cell survival, angiogenesis, invasion, and metastasis (24). Many studies have reported elevated expression of HuR in numerous malignancies (25, 26). HuR contains 3 RNA recognition motifs that bind to 3′-UTRs of mRNAs that bear AU- or U-rich sequences (27). In malignant tumors of the central nervous system such as glioblastoma, HuR has been linked to the augmented expression of genes such as TNF-α, interleukin (IL)-8, COX2, VEGF, TGF-β, and other genes involved in enhancing the tumorigenic phenotype, such as increased cell proliferation, evasion of apoptosis, angiogenesis, and invasion/metastasis (25, 26, 28). A more recent study showed that HuR promotes an antia apoptotic phenotype through the posttranscriptional control of Bcl-2, Mcl-1, and Bcl-xl, proteins belonging to the Bcl-2 family of antia apoptotic proteins (29).

In this study, we show that high expression of Msi1 in glioblastoma is partially triggered by HuR through its influence on Msi1 translation and mRNA stability, resulting in an increased steady-state level of MSI1 mRNA and increased Msi1 protein output. Supporting this idea, we observed that HuR and Msi1 have similar patterns of expression. Finally, we showed that Msi1 transgenic expression overcomes the impact of HuR knockdown on cell proliferation, apoptosis, and cell-cycle profile. In conclusion, we suggest that the HuR-Msi1 link is an important piece in gliomagenesis.

Materials and Methods

Cell culture

U251 and U343 glioblastoma cells were maintained in Dulbecco’s Modified Essential Medium (Thermo Scientific) supplemented with 10% FBS, penicillin, and streptomycin. HeLa cervical adenocarcinoma cells were maintained in Minimum Essential Medium (Thermo Scientific) supplemented with 10% FBS, penicillin, and streptomycin. Primary glioblastoma tumorspheres were obtained from surgically resected patient tumors and propagated in neurobasal media containing l-glutamine, N2 supplement (Gibco), B27 supplement (Gibco), heparin (Sigma), epidermal growth factor (EGF; PeproTech, Inc.), and basic fibroblast growth factor (bFGF; PeproTech, Inc.; ref. 30). For growth of primary glioblastoma cells as monolayers, the cells were cultured in the presence of Dulbecco’s Modified Essential Medium with 10% FBS, penicillin, and streptomycin. The U251 overexpression cell lines were previously established (22). U343 overexpression cell lines were created through stable G418 (Gibco) selection (800 μg/mL) after transfection of the pEF1/myc-His A plasmid (Invitrogen), which contains the expression cassette encoding for an EF-1α promoter-driven coding region for either Msi1 or GFP. After initial selection, 400 μg/mL of G418 was used for maintenance. The levels of Msi1 overexpression were monitored using reverse transcription quantitative PCR (qRT-PCR) analysis.

Immunoprecipitation of HuR ribonucleoprotein complexes

Immunoprecipitation of HuR ribonucleoprotein (RNP) complexes (31) was carried out using U251 glioblastoma cell lysates. Cell lysates were prepared and precleared using 15 μg of IgG isotype control and protein A-sepharose beads, preswollen in NT2 buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl₂, 5% bovine serum albumin, and 0.05% Nonidet P-40), for 30 minutes at 4°C. After preclearing, 100 μL of protein A-sepharose beads were incubated with 30 μg of anti-HuR antibody for 18 hours at 4°C then for 1 hour at room temperature with 3 mg of precleared cell lysate. After repeated washing and centrifugation of the sepharose beads with ice-cold NT2 buffer, HuR-bound RNA was released from protein using proteinase K and SDS. RNA was extracted using acid phenol–chloroform and ethanol precipitated. After reverse transcription (RT), qPCR analysis was carried out using gene-specific primers for MS11, GAPDH, and PTMA mRNAs.

In vitro transcriptions of biotinylated RNA and analysis of HuR bound to biotinylated RNA

A plasmid containing full-length Msi1 cDNA was used as a template for PCR. The PCR products were purified and
used as a template for in vitro transcription. In vitro transcription was catalyzed by T7 RNA polymerase, in the presence of biotin-CTP. Biotin pull-down assays were carried out by incubating 40 μg of cytoplasmic fractions with 1 μg of biotinylated transcripts for 1 hour at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal), and bound proteins were analyzed by Western blotting by using a mouse monoclonal antibody recognizing HuR. After secondary antibody incubations, signals were visualized by chemiluminescence.

**Reporter assay**

For luciferase assays, HeLa cells were cotransfected with the pSGG-MsI1 reporter vector and TAP-tagged HuR expression vector with Roche Fugene 6 transfection reagent (Roche Applied Sciences). An empty expression vector was used as a negative control. A total of 8 × 10^3 HeLa cells were plated in a 96-well culture plate 24 hours prior to transfection. Using the Fugene 6 reagent, 100 ng of the HuR expression vector (or empty vector for a negative control) and 1 ng of the UTR luciferase reporter vector were cotransfected. Forty-eight hours later, cell lysates were prepared with the Reporter Lysis Buffer (Promega). Using the Luciferase Assay Reagent (Promega), luminescence was read on a Berthold Technologies AutoLumat LB 953 Multi-Channel luminometer (Berthold Technologies).

**siRNA transfections**

U251 and U343 glioblastoma cells were reverse transfected with siRNAs with Lipofectamine RNAiMAX transfection reagent (Invitrogen). siRNAs targeting HuR were obtained from the Dharmacon RNAi Collection SMARTpool Line (Thermo Scientific). Negative control siRNA reagent was obtained from Thermo Scientific Dharmacon. U251 and U343 glioblastoma cells were transfected with siRNAs in 100-mm culture plates for Musashi1 and HuR protein analysis. Seventy-two hours posttransfection, cells were scraped from the plate and harvested for analysis.

**Quantitative RT-PCR**

Total RNA was extracted using the TRIzol reagent (Invitrogen). After lysis with TRIzol and separation of any RNA:protein complexes, total RNA was extracted into the aqueous phase using chloroform. Total RNA was precipitated out of the aqueous phase using isopropanol, washed with 75% ethanol, and resuspended in nuclease-free water (Ambion). The cDNA was synthesized using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random priming. Quantitative PCR was carried out using the TaqMan Gene Expression Master Mix (Applied Biosystems). All reactions were run on an ABI 7500 Real Time PCR machine (Applied Biosystems). ABI TaqMan Gene Expression Assay (Applied Biosystems) primer/probe sets were obtained for the ELAVL1, MSII, and ACTB mRNAs. The data were acquired using the ABI SDS 2.0.1 software package. ACTB mRNA was used as an endogenous control, and the data were analyzed using the 2^(-ΔΔCt) method.

**Western blot**

After collection of cells for analysis, cells were resuspended and sonicated in 2× SDS Laemmli sample buffer. A 10% SDS-PAGE gel with a 4% stacking gel was run in Tris-glycine-SDS buffer. A semi-dry transfer procedure was carried out onto a nitrocellulose membrane. After transfer, the membrane was blocked in TBS with Tween-20 and 5% milk. The membrane was probed with a rabbit monoclonal anti-Musashi1 antibody (Abcam), a mouse monoclonal anti-HuR antibody (Abcam), or mouse monoclonal anti-α-tubulin antibody (Sigma). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology) was used as a secondary antibody for Musashi1 or HRP-conjugated goat anti-mouse antibody (Zymed Laboratories) was used as a secondary antibody for HuR and α-tubulin. Electrochemiluminescence was used to detect the Musashi1, HuR, and α-tubulin protein using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Pierce Protein Products).

**mRNA stability assay**

U251 glioblastoma cells were reverse transfected with either the anti-HuR siRNA or scrambled negative control siRNA, in 35-mm plates at 50% confluency in antibiotic-free media. After 12 hours, the media were switched to media containing 5 μg/mL actinomycin D (Alexis Biochemicals) to inhibit transcription; each hour after that, total RNA was harvested using TRIzol (Invitrogen) following the manufacturer’s protocol. The cDNA was reverse transcribed using the ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan real-time PCR was carried out on the cDNA using primers (Applied Biosystems) for MSII and GAPDH mRNA. Quantitative PCR was carried out on an ABI 7500 Real Time PCR system (Applied Biosystems), and data were acquired using the ABI SDS 2.0.1 Software Package (Applied Biosystems) and analyzed using the 2^(-ΔΔCt) method. Subsequently, the data were fitted to a linear regression, and the regression was used to calculate the half-life of the MSII mRNA.

**Polysomal gradient preparation and analysis**

HuR siRNAs, or corresponding negative control siRNA, were reverse transfected into U251 glioblastoma cells. Seventy-two hours after transfection, translation was arrested using 0.1 mg/mL of cycloheximide. Cells were dissociated using trypsin, and cell pellets were formed. Pellets were resuspended and lysed in polysome extraction buffer (20 mmol/L Tris-HCl, pH 7.5, 100 mmol/L KCl, 5 mmol/L MgCl2, 0.3% Igepal CA-630, protease inhibitors, and 0.1 mg/mL cycloheximide). After centrifugation to remove insoluble material, the lysate was overlaid on a 10% to 50% sucrose gradient in a buffer solution of 10% mol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 5 mmol/L MgCl2. After ultracentrifugation at 39,000 rpm at 4°C, 1-mL fractions were obtained on a density gradient fractionation system (Brandel). A254 was measured during the entire fractionation process. Total RNA was extracted from
Quantitative RT-PCR of clinical glioblastoma samples

Previously characterized GBM specimens were used for this study (32). Briefly, tissue samples were obtained from the solid tumor, minced, and enzymatically dissociated. Primary tumor cell cultures were maintained under serum-free conditions according to Lee and colleagues (33). Clinical glioblastoma samples were prepared for total RNA using the RNeasy Mini Kit (Qiagen). cDNA was prepared using the ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and real-time PCR reactions were carried out with TaqMan Gene Expression assay probes (Applied Biosystems) for MSI1, ELAVL1, and ACTB mRNA. The data were acquired using the ABI SDS 2.0.1 Software Package (Applied Biosystems) and analyzed using the 2^(-ΔΔCt) method. The qRT-PCR measurements of HuR/Msi1 were carried out in triplicate and averaged for each tumor sample (n = 20) and the control tissue sample U251. The Ct value of these genes was normalized by subtracting the Ct of the endogenous control ACTB mRNA measurement.

Tissue microarray and analysis

Immunohistochemistry was conducted on the GL806 brain glioblastoma and normal tissue array, which contains 35 cases of glioblastoma, 5 normal tissue, duplicated cores per case (US Biomax Inc.). Antigen retrieval was conducted by heat induction of the array slides. Monoclonal anti-HuR antibody (Molecular Probes Inc.) and polyclonal anti-Msi1 antibody (R&D Systems, Inc.) were used for immunohistochemical staining of the tissue arrays.

All stained slides were scanned at 40× using an Aperio CS Scanscope (Aperio Technologies) and quantified using the available Aperio algorithms. Immunodetection of Msi1 or HuR was quantified according to percentage of cells stained. The percentages of stained cells were multiplied by 3 for intensively stained cells, moderately stained cells by 2, and mildly stained cell by 1 and used as a staining index for the quantitative assessment of changes in marker expression in each treated group, as previously reported (34).

Cell proliferation assay

Cell proliferation was conducted on the U251 Msi1 or GFP overexpression cell lines (U251:MSI1 or U251:GFP, respectively) and U343 Msi1 or GFP overexpression cell lines (U343:MSI1 or U343:GFP, respectively). Either HuR siRNA or control siRNA was transfected into each cell line. After 48 hours of transfection, cell proliferation was assessed with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega). After 1 hour, absorbance at 490 nm was measured on a Synergy HT Multi-Mode microplate reader (Biotek).

Cell-cycle analysis

Cells were prepared for cell-cycle distribution analysis using propidium iodide staining of DNA content. Cells were harvested using trypsin, pelleted via centrifugation at 1,000 rpm for 3 minutes at 4°C, and resuspended in ice-cold PBS. The cells were pelleted once again and resuspended in 500 µL of ice-cold PBS. The resuspended cells were then slowly dropped into a vortexing tube containing 9 × volume of ice-cold 100% ethanol for fixation. The solution was incubated at 4°C for 30 minutes. The cells were then pelleted via centrifugation, washed with PBS once more, and resuspended in the propidium iodide staining solution, which is composed of a solution of PBS containing 0.1% (v/v) of Triton-X-100 (Sigma-Aldrich), 200 µg/mL of RNase A (Sigma-Aldrich), and 20 µg/mL of propidium iodide (Invitrogen). The cells were incubated in staining solution in the dark for 30 minutes at room temperature. The cells were analyzed on the BD FACSCanto Flow Cytometry Machine (BD Biosciences). Flow cytometric data were analyzed using ModFit LT (Verity Software House) to calculate cell-cycle distribution.

Caspase-3/7 activity assay

Apoptosis was assayed with the U251 Msi1 or GFP overexpression cell lines (U251:MSI1 or U251:GFP, respectively) and U343 Msi1 or GFP overexpression cell lines (U343:MSI1 or U343:GFP, respectively). Either HuR siRNA or control siRNA was transfected into each cell line. After 48 hours of transfection, apoptosis was monitored with the Caspase-Glo 3/7 Assay Reagent (Promega). Luciferase measurements were acquired with the SpectraMax M5 Multi-Mode Microplate Reader (Molecular Dynamics).

Statistical analysis

All statistical analysis was conducted with the Student t test. Data are presented as the mean ± SEM.

Results

Identification of U- and AU-rich elements in the Msi1 3′-UTR

The MSI1 mRNA contains a long 3′-UTR, suggestive of posttranscriptional regulation. In a previous report, our laboratory has identified 5 tumor suppressor miRNAs that modulate Msi1 expression (22). Upon closer observation, we also identified regions in the Msi1 3′-UTR containing potential uracil-rich elements amenable for regulation by HuR (ref. 35; Fig. 1A). In agreement with the idea that HuR functions as a positive regulator of Msi1, HuR has been shown to be upregulated in many tumors (24) including glioblastoma, where Msi1 is also highly expressed.

Immunoprecipitation of HuR enriches for the MSI1 mRNA

First, we conducted an immunoprecipitation of HuR ribonucleoprotein complexes (RNP-IP) in U251 glioblastoma cell lines followed by qRT-PCR to determine whether or not the MSI1 mRNA is associated with HuR protein. In 3 biological replicates, we observed an overall enrichment of approximately 20-fold of the MSI1 mRNA in immunoprecipitation carried out with anti-HuR antibodies in comparison to the GAPDH mRNA, which does not bind to HuR.
Figure 1. Discovery of HuR as a trans-acting posttranscriptional regulatory factor for Msi1. A, a schematic of the Msi1 mRNA with the 3′-UTR shown in the inset. The nucleotide tracts bolded, italicized, and underlined indicates the putative HuR-binding sites. B, immunoprecipitation of RNA:HuR protein complexes. E, in immunoblot. A beads only control was included to account for any nonspecific interaction. The RNA recovered was reverse transcribed and qPCR was carried out on the cDNA. Primers were used for GAPDH, PTMA, and Msi1 mRNAs. GAPDH mRNA is used as a negative control and PTMA mRNA is a positive control. C, diagram of the mRNA fragments used in biotin pull-down experiment. Three fragments were made, one for the coding region (CDS) and two for each half of the 3′-UTR. D, biotin pull-down assay confirms HuR interaction with Msi1 mRNA. Biotinylated fragments of the Msi1 mRNA were in vitro transcribed. Three fragments of the mRNA were analyzed, one for the coding region and two for each half of the 3′-UTR. The GAPDH 3′-UTR was included as a negative control. After incubation of the fragments with streptavidin beads, the protein was recovered by pull-down analysis and immunoblotted for HuR. A beads only control was included to account for any nonspecific interaction and a "lysate" was included as a positive control for the immunoblot. E, influence of HuR on Msi1 protein expression is dependent on the 3′-UTR. The Msi1 3′-UTR was cloned downstream of a PEST-destabilized luciferase gene (luc2P). The luciferase construct was cotransfected with a TAP-tagged HuR expression vector (denoted as TAP-HuR) in HeLa cervical adenocarcinoma cells. An empty vector (denoted as TAP) was used as a negative control. Data were analyzed with the Student t test and are presented as the mean ± SEM. Experiment was carried out in triplicate. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

As a positive control for HuR binding, the mRNA encoding prothymosin-α, an antiapoptotic protein, was evaluated (36). HuR binding to Msi1 mRNA seems to be even stronger than HuR binding to prothymosin-α mRNA (Fig. 1B).

To map the region of the Msi1 mRNA that contains the HuR-binding site, we carried out biotin pull-down experiments with biotinylated fragments of the Msi1 mRNA. Three fragments were made, one for the coding region of Msi1 and two for the 3′-UTR (a proximal region and a distal region; Fig. 1C). The GAPDH 3′-UTR was included as a negative control. After incubation with U251 cell lysate, the bound protein eluate was immunoblotted for HuR. The results of the pull-down Western blot experiments show a strong HuR signal for the distal half of the Msi1 3′-UTR than for the beads only (Fig. 1D). A weak but positive signal is seen for the proximal region, indicating that indeed binding exists, but most likely with low affinity. "and biotinylated RNA" can be removed as the fragments referred to in the beginning of the sentence refers to the biotinylated RNA. The coding region fragment shows no signal, indicating a lack of a binding of HuR to this region of the Msi1 mRNA.

Luciferase reporter assay confirms interaction with Msi1 3′-UTR

To further examine the effect of HuR on Msi1 expression, a luciferase reporter carrying the Msi1 3′-UTR was constructed and tested. The luciferase coding region contained a PEST degradation sequence (37) that effectively reduces the luciferase protein half-life to approximately 1 hour, thus reducing the chances of any ambiguous results due to protein accumulation. Compared
with the empty expression vector, we detect a 2-fold increase \((P = 2.5 \times 10^{-7})\) in luciferase activity when we cotransfect a HuR expression vector (Fig. 1E), thus confirming the interaction of HuR with the Msi1 3′-UTR.

**HuR silencing suggests that HuR positively regulates Msi1 expression**

To further validate the regulation of Msi1 by HuR, its levels were reduced by using HuR-directed siRNA in U251 and U343 glioblastoma cells. A significant reduction in HuR (U251: \(P = 1.5 \times 10^{-3}\); U343: \(P = 1.3 \times 10^{-3}\)) was followed by a concomitant reduction in Msi1 expression at both the mRNA (\(P = 3.4 \times 10^{-7}\); U343; \(P = 5.1 \times 10^{-3}\); Fig. 2A and B) and protein levels (Fig. 2C and D), suggesting that HuR positively regulates Msi1 expression in glioblastoma cells.

**HuR increases MSI1 mRNA stability**

To dissect the mechanism by which HuR controls Msi1 expression, we first measured the stability of MSI1 mRNA (38). We knocked down the expression of HuR using siRNAs and 12 hours posttransfection, we treated the cells with 5 μg/mL of actinomycin D, an inhibitor of RNA polymerase elongation. After transcription was inhibited, mRNA decay was measured without interference. The mRNA decay rate of the MSI1 mRNA was measured in the presence and absence of the HuR RBP. Over the course of 3 hours, we observed a faster rate of decay in HuR-silenced cells than in control cells. We fitted the data to a linear regression model and used the model to calculate the half-life of the MSI1 mRNA. The half-life of the MSI1 mRNA is 8.17 hours, whereas in HuR-silenced cells, the MSI1 mRNA half-life was reduced to approximately 3.12 hours, representing a 62% increase in the rate of decay \((P = 4.8 \times 10^{-5})\) of the MSI1 mRNA (Fig. 3).

**HuR positively regulates Msi1 translation**

In addition to mediating mRNA stability, HuR can also modulate translation (24). To determine whether HuR also affects the translation of MSI1 mRNA, we conducted polysome fractionation analysis using extracts from U251 control and HuR-knockdown cells (39). In this assay, polysomes, which contain actively translating mRNAs and have high sedimentation rates, are partitioned from untranslated mRNAs, which have low sedimentation rates (Fig. 4A and B). Fine fractions are acquired from the gradients and levels of mRNAs can be measured with Northern blot or qRT-PCR. The results showed that the profile of the GAPDH mRNA, encoding a housekeeping protein, was

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**Figure 2.** MSI1 mRNA and protein levels are dependent on HuR levels. A and B, MSI1 mRNA levels are upregulated by HuR. Seventy-two hours after siRNA-mediated silencing of HuR (50 nmoL/L) in U251 (A) and U343 (B) glioblastoma cells, MSI1 mRNA was quantified by reverse transcription followed by qPCR analysis; β-actin mRNA was used as an endogenous control and the data were analyzed using the \(2^{-\Delta\Delta Ct}\) method. Data were analyzed with the Student t-test and are presented as the mean ± SEM. Experiment was carried out in triplicate. C and D, Msi1 protein is positively dependent on HuR levels. By 72 hours after siRNA-mediated silencing of HuR (50 nmoL/L) in U251 (C) and U343 (D) glioblastoma cells, Msi1 protein levels were assessed by Western blot analysis. α-Tubulin served as a loading control.

**Figure 3.** HuR stabilizes the MSI1 mRNA. The stability of the MSI1 mRNA mediated by HuR was assessed in U251 glioblastoma cells. siRNAs (50 nmoL/L) directed against HuR were transfected into U251. A scrambled siRNA was used as a negative control. Actinomycin D (5 μg/mL) was added to the cell media 12 hours after siRNA transfection. Total RNA samples were obtained, and MSI1 mRNA levels were determined using qRT-PCR. The data were analyzed using the \(2^{-\Delta\Delta Ct}\) method and the GAPDH mRNA was used as an endogenous control. Relative MSI1 mRNA levels were plotted against time after actinomycin D addition and fitted to a linear regression model. MSI1 mRNA half-life was calculated using the linear regression model. Data were analyzed with the Student t-test and are presented as the mean ± SEM. Experiment was carried out in triplicate.
HuR Regulation of Musashi1

Figure 4. HuR promotes translation of the MSI1 mRNA. Sucrose gradients were used to fractionate lysates from control U251 cells and cells with HuR knockdown. After fractionation of the sucrose gradient post-ultracentrifugation, RNA from each fraction was extracted with TRIzol and analyzed by real-time PCR. GAPDH mRNA, a transcript that is not regulated by HuR, was included as a negative control. A and B, the results of the A254 during fractionation. The first 3 peaks from the left are the 40S, 60S, and 80S subunits, whereas the peaks to the right are the heavier polysomal fractions. C and D, the percentage distribution of the GAPDH (C) or MSI1 (D) is shown. Upon HuR silencing, the distribution of MSI1 mRNA shifts from the heavier polysomes to the lighter polysomal fractions while GAPDH mRNA is unaffected. The results of this experiment suggest that HuR promotes the translation of the MSI1 mRNA.

unaffected by HuR silencing (Fig. 4C), whereas we observed a shift in MSI1 mRNA from heavier polysomal fractions to the lighter fractions when comparing control cells with HuR-knockdown cells (Fig. 4D). This result indicates that HuR promotes Msi1 expression by a combination of increasing MSI1 mRNA stability and enhancing its translation.

HuR and Msi1 expression are positively correlated

It has been suggested that Msi1 is required to maintain the "cancer stem cell population" (18, 40). In support of this idea, previous studies done in our laboratory and others (30) showed that tumor cells maintained as spheroids, a condition that favors the growth of cells with "stem-like" characteristics, have increased Msi1 expression compared with cells grown as adherent monolayers. We tested to see whether the same applies to HuR. HuR expression was measured by qRT-PCR in 2 tumor lines established at University of Texas Health Science Center at San Antonio revealing also that as with Msi1, HuR expression levels are higher in spheroid cultures relative to monolayers (Fig. 5A).

We then analyzed the mRNA expression of HUR and MSI1 in clinical samples of glioblastoma by qRT-PCR. When analyzed as on a scatter plot, a statistically significant ($P = 0.0201$) positive correlation of HUR and MSI1 mRNA expression is observed [Pearson correlation score = 0.503, 95% confidence interval (CI), 0.09–0.77], indicating that glioblastoma tumors that display high Msi1 expression also express high levels of HuR (Fig. 5B).

We extended our expression analysis to examine the protein expression of both HuR and Msi1. We immunostained with both anti-Msi1 and anti-HuR antibodies a tissue microarray containing 35 high-quality glioblastoma cores and 5 normal brain tissue cores in duplicate (Fig. 5C). We observed that although ubiquitously expressed, HuR is more abundant in glioblastoma than in normal brain tissue (Fig. 5D, tumor average score = 83.48, SEM = 1.507, 95% CI, 80.47–86.50 vs. normal tissue average score = 61.00, SEM = 9.452, 95% CI, 39.62–82.38, $P < 0.0001$). As for Msi1, whereas no expression was detected in normal brain, which is consistent with the notion that Msi1 is not present in differentiated cells (3), Msi1 expression was often detected in glioblastoma samples (Fig. 5D and E, tumor average score = 41.14, SEM = 4.017, 95% CI, 33.11–49.16 vs. normal tissue average score = 0.0, SEM = 0.0, 95% CI, 0.0–0.0, $P < 0.0001$). From this analysis, we also ascertain that both Msi1 and HuR expression positively correlate in glioblastoma (Pearson correlation = 0.497, $P < 0.0001$).

Ectopic Msi1 expression rescues cell proliferation inhibition by HuR silencing

We sought to determine whether Msi1 is a main mediator of HuR function in glioblastoma cells. We silenced
Figure 5. HuR and Msi1 expression levels correlate positively. A, MSI1 mRNA and HUR mRNA levels are higher in tumor cells with "stem cells" characteristics. HuR and Msi1 expression were assessed in 2 primary glioblastoma samples grown as spheroids versus monolayers. The ACTB mRNA was used for normalization when using the $2^{-\Delta\Delta C_t}$ method for mRNA relative quantification. Data were analyzed with the Student t test and were presented as the mean ± SEM. Experiment was carried out in triplicate. B, HUR and MSI1 mRNA expression positively correlates in GBMs. qRT-PCR was carried out on total RNA extracted from the clinical samples and the data were analyzed using the $2^{-\Delta\Delta C_t}$ method. The ACTB mRNA was used as an endogenous control and all samples were compared with the U251 glioblastoma cell line. The $\Delta\Delta C_t$ Msi1 was plotted on the abscissa, and the $\Delta\Delta C_t$ HuR was plotted on the ordinate. A linear regression model was generated with a correlation score of 0.503 and P value of 0.0201; thus, HuR and Msi1 exhibit statistically significant linear correlation in expression. C, representative microscopic images (40×) of immunohistochemistry of Msi1 and HuR in a glioblastoma tissue microarray. D, quantifying glioblastoma tissue microarray stainings of HuR and Msi1. Staining scores for HuR and Msi1 in glioblastoma (35 cores) was averaged and compared with the average score for staining in normal brain tissue (5 cores). Both HuR and Msi1 are more expressed in glioblastoma than in normal brain tissue (P < 0.0001). Data were analyzed by the Student t test and presented as the mean ± SEM. E, individual scores of each glioblastoma and normal brain tissue samples for HuR and Msi1 are graphed on a vertical scatter plot. In glioblastoma, HuR had an average score of 83.48, SEM = 1.507 with a 95% CI ranging from 80.47 to 86.50, whereas in normal brain tissue, HuR had an average score of 61.00, SEM = 9.452 with a 95% CI ranging from 39.62 to 82.38. In glioblastoma, Msi1 had an average score of 41.14, SEM = 4.017 with a 95% CI ranging from 33.11 to 49.16, whereas in normal brain tissue, Msi1 had an average score of 0.0, SEM = 0.0 with a 95% CI ranging from 0.0 to 0.0.
HuR via siRNA in control lines and in lines containing a MSI1 transgene that does not contain the 3′-UTR, rendering the ectopic Msi1 expression immune to any posttranscriptional regulation. 48 hours after transfection, we measured cell proliferation by MTS assay. While HuR knockdown caused a reduction in cell proliferation in control cells (31% reduction in U251 malignant glioma cells and 21% reduction in U343 malignant glioma cells, Fig. 6A and B), the effect was partially ameliorated in cells with transgenic Msi1 expression (13% reduction in U251 and 2.7% in U343, Fig. 6A and B). This result suggests that the impact of HuR on cell proliferation is partially mediated by Msi1.

Ectopic Msi1 expression attenuates the effect of HuR silencing on cell-cycle distribution

We further determined whether the impact HuR has on the cell cycle is partially mediated by Msi1. Experiments were carried out with the U251 and U343 Msi1 and GFP control transgenic cell lines described above. Depletion of HuR by siRNAs caused an impact on cell-cycle distribution in both U251 and U343 cells. In U251 cells, HuR silencing promoted a distribution of cells moving from G1 to S-phase in U251:GFP cells (Fig. 7A), whereas in U343 cells, we observed an expansion of the population in G2 phase with a concomitant decrease of cells in G1 and a slight decrease in cells found in S-phase (Fig. 7B). When HuR silencing was conducted in U251 and U343 cells which ectopically express Msi1, the magnitude of the effect of HuR siRNA on the distribution of cells in each cell-cycle phase is blunted (Fig. 7A and B). In conclusion, our results indicate that HuR has a profound effect on cell-cycle distribution and suggests that this effect is partially achieved via Msi1.

Ectopic Msi1 expression rescues apoptosis induced by HuR silencing

Because HuR and Msi1 have been implicated in the promotion of a prosurvival/antiapoptotic program in the cell (18, 19, 29, 36, 41–43), we sought to determine whether HuR and Msi1 cooperatively function to promote an antiapoptotic phenotype. When HuR expression is depleted by siRNA transfection, caspase-3/7 activity is increased 3.3-fold in U251:GFP control cells and 3.9-fold in U343:GFP control cells, Fig. 8A and B, as compared with the control siRNA transfection. However, when HuR is depleted in either the U251:MSI1 or U343:MSI1 stable cell lines, caspase-3/7 activity is only induced 2.3-fold in U251:MSI1 cells and 3.2-fold in U343:MSI1 (Fig. 8A and B), suggesting that Msi1 transgenic expression curtails the induction of apoptosis when HuR is silenced. This result indicates that the antiapoptotic phenotype triggered by HuR is partially mediated by Msi1.

Discussion

In this study, we explored the posttranscriptional regulation of the Msi1 expression by HuR. We found that HuR binds to sites located in the distal portion of the MSI1 3′-UTR and controls its expression via mRNA stabilization and translational upregulation. We determined that HuR and Msi1 expression levels are positively correlated and that the identified regulation has potentially important implications for glioblastoma cell growth.

RBPs regulate mammalian gene expression at the posttranscriptional level, mediating many processes such as mRNA maturation, splicing, transport, storage, stability, and translation. TTR-RBPs (44) are a large family of transacting factors that control mRNA stability and translation through binding to cis-regulatory elements encoded by an mRNA. Many TTR-RBPs are present in the cell and likely function jointly on a particular mRNA, through additive, antagonistic, or synergistic actions. The role of pathologic and aberrant actions of RBPs in many disease processes, including cancer, has been revealed in recent years. One RBP of particular interest in cancer is HuR (HuA), a member of the Hu/ELAV family of RBPs that also encompasses the neuronal proteins HuB, HuC, and HuD. Through interactions with specific subsets of mRNAs, HuR can potentiate...
biological features of cancers such as sustained proliferation, evasion of apoptosis, increased angiogenesis, and invasion/metastasis (24).

HuR is a ubiquitous, multifaceted RBP implicated in mRNA splicing, translation regulation, and mRNA stabilization (24). In recent years, HuR has been implicated as a potent factor in tumorigenesis (24) through its ability to influence many characteristics of cancer cells (45, 46) including limitless proliferation, sustained angiogenesis, tissue invasion, and metastasis, via its interactions with cancer-related genes [e.g., c-Myc (47), p27 (48), VEGF (49), and Snail (50)]. In high-grade gliomas, prominent HuR expression has been shown; specific mRNA ligands, such as VEGF, COX2, IL-6, IL-8, TGF-\(\beta\), and TNF-\(\alpha\) mRNAs, have been identified (25, 26, 28), but the molecular characterization of the interaction of HuR with these ligands has yet to be carried out. These examples of posttranscriptional regulation by HuR suggest that glioma cell growth necessitates the use of HuR to maintain and augment the growth of glioma cells (26).

Our study explores a more global effect by HuR in glioblastoma, through HuR’s positive regulation of another oncogenic RBP, Msi1, previously implicated in controlling a wide subset of mRNAs with cancer-related functions in proliferation, differentiation, survival, and apoptosis (51). Our findings posit that HuR not only elicits direct effects on cancer cell gene expression but also can exert indirect gene-regulatory effects by controlling expression of another key regulator of gene expression, Msi1. Our findings may be extended to other malignancies that depend on Msi1.

**Figure 7.** Cell-cycle distribution is altered upon HuR silencing with siRNAs and is partially attenuated with ectopic Msi1 expression. Cell-cycle analysis was conducted using propidium iodide staining of ethanol-fixed cells for DNA content which was analyzed using flow cytometry. A and B, U251:GFP control and U251:MSI1 cells were transfected with either HuR siRNA or scrambled control siRNA. A similar experiment was carried out in U343:GFP and U343:MSI1 cells. Cell-cycle distribution was analyzed using the ModFit LT Software Package. Then, the percentage of cells in each phase found in the HuR siRNA experiment was subtracted from the control siRNA experiment. HuR silencing caused a large shift in cell-cycle distribution in the U251:GFP (A) and U343:GFP (B) cells. However, when Msi1 is ectopically expressed, the change in cell-cycle distribution induced by HuR silencing was attenuated (A and B). Data were analyzed by the Student t test and presented as the mean \(\pm\) SEM. Experiment was carried out in triplicate.

**Figure 8.** Ectopic expression of Msi1 partially recovers the effect of HuR silencing on apoptosis. A, HuR or control siRNAs were transfected in U251:GFP and U251:MSI1 cells. B, similar experiments were carried out in U343:GFP and U343:MSI1 cells. Apoptosis was measured 48 hours posttransfection using a luciferase-based reagent to monitor caspase-3/7 activity. HuR silencing was able to induce apoptosis, whereas Msi1 ectopic expression partially inhibits this effect. Data were analyzed by the Student t test and presented as the mean \(\pm\) SEM. Experiment was carried out in triplicate.
The HuR:Msi1 relationship discussed in this article creates a positive cascade network motif (56) where the cellular logic for gliomagenesis requires HuR to essentially "activate" Msi1 gene expression. Moreover, HuR also autoregulates itself bolstering its own production in a positive feedback loop (57–59). The interaction between these 2 RBPs creates a 2-component composite gene regulatory network. In Saccharomyces cerevisiae, 21 of the 46 studied RBPs are a 2-component composite gene regulatory network. In

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Received May 8, 2011; revised October 9, 2011; accepted October 28, 2011; published online January 18, 2012.

Table 1. Summary of malignancies that exhibit increased HuR and Msi1 expression

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>HuR</th>
<th>Msi1</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>↑</td>
<td>↑</td>
<td>(12, 25, 64)</td>
</tr>
<tr>
<td>Medulloblastoma</td>
<td>↑</td>
<td>↑</td>
<td>(18, 25)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>↑</td>
<td>↑</td>
<td>(8, 31)</td>
</tr>
<tr>
<td>Cervical adenocarcinoma</td>
<td>↑</td>
<td>↑</td>
<td>(9, 52)</td>
</tr>
<tr>
<td>Non–small cell lung cancer</td>
<td>↑</td>
<td>↑</td>
<td>(10, 53)</td>
</tr>
<tr>
<td>Colon cancer</td>
<td>↑</td>
<td>↑</td>
<td>(19, 59)</td>
</tr>
</tbody>
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NOTE: ↑ indicates increased expression. References for each study are called out in the rightmost column.

However, posttranscriptional genetic regulatory network motifs are not limited by RBP nodes but extend to microRNAs. A recent study from our laboratory illustrates the posttranscriptional regulation of Msi1 by tumor suppressor miRNAs (22). Moreover, we also recently showed that 25% of HuR-binding sites in 3′-UTRs mapped by CLIP (cross-linking and immunoprecipitation) coincide with miRNA predicted sites, suggesting an interplay between RBPs and miRNAs (60). These complex relationships show the nonlinearity of posttranscriptional network entities and shows that their integration creates a larger, complex network that contains the pertinent logic for the molecular pathogenesis in glioblastoma. Furthermore, posttranscriptional gene networks are intimately intertwined with the transcriptional gene network thus an inclusive analysis of the circuitry required for glioblastoma tumorigenesis surfaces, providing insights into glioblastoma cellular logic and physiology and the emergence of genetic network–directed therapies (61–63).

Disclosure of Potential Conflict of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Suzanne C. Burns for critical reading and comments on the manuscript and the anonymous referees for their critical reading and suggestions for the manuscript throughout the review process.

Grant Support

K. Abdelmohsen, J.L. Martinadle, K. Tominaga, and M. Gorospe are supported by the Intramural Research Program of the National Institute on Aging, NIH. V. Patel is supported by the Intramural Research Program at the National Institute of Dental and Craniofacial Research, NIH. D.T. Vo, M. Qiao, T.L. Burton, and L.O.F. Penalva are supported by grants from the Children’s Brain Tumor Foundation, Association for Research of Childhood Cancer, and The Max and Minnie Tomerlin Voelcker Fund. A. J. Brenner is supported by the Cancer Therapy & Research Center P30 Cancer Center Support Grant from the National Cancer Institute (CA054174).

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The Oncogenic RNA-Binding Protein Musashi1 Is Regulated by HuR via mRNA Translation and Stability in Glioblastoma Cells

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