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Loss of Repression of HuR Translation by miR-16 May Be Responsible for the Elevation of HuR in Human Breast Carcinoma

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ABSTRACT

Elevated levels of RNA binding protein HuR were found in various human cancers. However, the mechanisms underlying HuR over-expression in cancers have not been fully elucidated. Here, we show that miR-16 acts as a novel post-transcriptional regulator for HuR. Knockdown of miR-16 increased HuR protein levels in MDA-MB-231 cells, while over-expression of pre-miR16 reduced HuR expression. Neither knockdown nor over-expression of miR-16 could alter the mRNA levels of HuR. Instead, knockdown of miR-16 increased the level of de novo synthesized HuR protein. Importantly, mechanistic studies showed that miR-16 associated with the 3′UTR of HuR, and knockdown of miR-16 markedly increased the luciferase activity of a HuR 3′UTR-containing reporter. We further demonstrate that the level of miR-16 was inversely correlated with HuR protein level in human breast carcinoma. Together, our results suggest an important role of miR-16 in regulating HuR translation and link this regulatory pathway to human breast cancer. J. Cell. Biochem. 111: 727–734, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: miR-16; HuR; TRANSLATIONAL REGULATION; BREAST CARCINOMA

The contribution of post-transcriptional gene regulatory events such as mRNA turnover and translation in human cancers is becoming increasingly apparent. Involvement of RNA binding proteins (RBPs) including HuR, TTP, AUF1, CUGBP-2, and TIAR in post-transcriptional regulation during human cancers has been intensively reported [Denkert et al., 2004a; Gouble et al., 2002; Suswam et al., 2005, 2008; Natarajan et al., 2008]. These RBPs may act either directly to alter translational efficiency or serve as regulators for the degradation of the transcripts targeted. Among them, the role of HuR has attracted most of the attention. As a ubiquitously expressed member of Hu RBP family, HuR regulates the stability, translation, and nuclear export of mRNAs bearing AU- and U-rich elements (AREs) in their 3′-untranslated regions (3′UTR) [Wang et al., 2000; Mazan-Mamczarz et al., 2003; Sengupta et al., 2003; Yi et al., 2010]. Thus far, a variety of tumor-related transcripts include those encoding cell-cycle regulators, such as cyclins A, B1, E [Wang et al., 2003; Guo and Hartley, 2006], proliferation-associated genes, such as c-myc, c-fos, and p53 (9) [Mazan-Mamczarz et al., 2003; Wang et al., 2003; Kim et al., 2009], as well as factors controlling tumor growth, such as VEGF, COX-2, and TNF-α [Levy et al., 1998; Dean et al., 2002; Sengupta et al., 2003], have been identified as targets of HuR.

Although HuR is predominantly localized at the nucleus, it is well accepted that the presence of HuR in the cytoplasm determines HuR's ability to regulate mRNA stability and translation. For example, elevation of cytoplasmic HuR in various cancers, such as breast cancer, ovarian carcinoma, and colon carcinoma, is linked to the stabilization of mRNAs encoding cancer-related genes such as COX-2, VEGF, β-actin, etc., and correlates with the tumor grade in human breast and colon cancers as well as with poor outcome in human ovarian carcinoma [Dixon et al., 2001; Erkinheimo et al., 2003; Denkert et al., 2004a,b; Dormoy-Raclet et al., 2007]. The

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mechanisms that control cytoplasmic presence of HuR are complex. The nucleocytoplasmic shuttling sequence (HNS) located within the hinge region of HuR is of critical importance for its shuttling. In the nucleus, HuR interacts with proteins SETα, SETβ, pp32, and APRIL. The nuclear export of HuR was shown to involve the association of HuR with two of its nuclear ligands, pp32 and APRIL [Brennan et al., 2000]. Cell signaling events may also control the distribution of HuR between nucleus and cytoplasm. For examples, the kinases AMPK (AMP-activated protein kinase) and Cdk1 (cyclin-dependent kinase 1) have been shown to regulate the cytoplasmic presence of HuR by a different mechanism, and in turn influence the stability of mRNAs that encode cyclin A, cyclin B1, c-fos, and SIRT1 in cell division or replicative senescence [Wang et al., 2003; Abdelmohsen et al., 2007]. However, the significance of these signal elements in human cancers has not been fully demonstrated.

The elevation of HuR in human cancers leads to higher cytoplasmic levels, which in turn increases COX-2 expression through stabilizing the COX-2 mRNA [Erkinheimo et al., 2003; Denkert et al., 2004b]. Therefore, investigation into the mechanisms underlying the regulation of HuR is certainly critical for better understanding the onset and development of human cancers. In this regard, miR-519 and miR-125a have been shown to interact with the coding region or 3′UTR of HuR mRNA and repress HuR translation in colon cancer as well as in breast cancer cells [Abdelmohsen et al., 2008; Guo et al., 2009]. Given that the elevation of HuR in human cancers is tremendous but the effects of miR-519 or miR-125a are moderate, it is plausible to postulate that other factors may also involve in the regulation of HuR as well.

In this study, we describe miR-16 as a novel regulator of HuR translation. We discovered that miR-16 interacted with HuR mRNA in the 3′-UTR and repressed HuR translation. The reduction of miR-16 was accompanied with the elevation of HuR in human breast cancer. Our study highlighted a potential role of miR-16 in the regulation of HuR in human breast cancer.

MATERIALS AND METHODS

CELL CULTURE, ANTISENSE microRNA, AND TRANSFECTION

MDA-MB-231 human breast cancer cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 5% CO_2. The miR-125b antisense and control antisense were from Ambion and transfected by oligofectamine (Invitrogen) following the manufacturer's instructions. All plasmids were transfected using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were collected 48–72 h after transfection for further analysis.

ANALYSIS OF THE INTERACTION BETWEEN miR-16 AND HuR mRNA

cDNA was used as a template for PCR amplification of HuR mRNA fragments. All 5′ primers contained the T7 promoter sequence CCAAGCTTCTAATACGACT CACTATAG. To prepare fragment CR spanning both coding region and 3′ UTR (positions 192–1650), 5′-(T_7) ATGGCCGAAGACTGCAGGGGTGAC-3′ and 5′-ACG GGACCTGCCTGGAAAAGGA-3′ were used. To prepare the HuR 3′-UTR fragments A (positions 1645–2550), 5′-(T7) CCCGTTGCCACCTCCTGCTCAC-3′ and 5′-CCTTCCTCCGGGCTCCTGGTTTA-3′ were used. For miR-16-HuR mRNA interaction assays, PCR-amplified HuR fragments CR and A were used as templates to transcribe biotinylated transcripts by using T7 RNA polymerase in the presence of biotin-UTP, as described previously [Wang et al., 2000]. Poly(A) polymerase was used to add a biotinylated poly(A) tail to the miR-16 RNA in the presence of biotin-ATP. One microgram of purified biotinylated HuR or 1 μg of purified biotinylated miR-16 transcripts was incubated with 30 μg of cytoplasmic extracts (for pull down of miR-16) or 5 μg of total RNA (for pull down of HuR transcripts) in a 50 μl reaction mixture containing 25 μl HeLa cell S-100 extract, 1 mM ATP, 0.2 mM GTP, 40 U/ml RNasin, 5 mM EGTA, 30 μg/ml creatine kinase, 25 mM creatine phosphate for 60 min at room temperature. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo) that had been preblocked with S-100 extracts, and the pull-down material was analyzed by RT-PCR.

PCR ANALYSIS

For RT-PCR analysis, primers TTCACATCCGATTCAGCC and TCTACTGCCATCATTACACG for HuR mRNA, primers GATTACCAGGGATTTCAGT and GACACCTTTAGGCAGACC for luciferase mRNA, primers GAAACGGATGATAACTGG and TCGCCATAAATAAGAAGAG for Renilla luciferase mRNA, and primers 5′-CGAGTCAACGGATTTGGTGGTAT-3′ and 5′-AGCCTTCTCCATGGTGAA GAC-3′ for GAPDH mRNA were used. For real-time PCR to analyze the levels of HuR, COX-2, c-fos, and SIRT1 mRNAs, primers 5′-GCCTGTTCAGCAGCATTG-3′ and 5′-GGCGAGCATACGACACCTTA-3′ for HuR, primers 5′-GCCTGATGATTG CCCGACTCC-3′ and 5′-TGTGTTCCCGCAGCCAGATTG-3′ for COX-2, primers 5′-GTCTCCAGTGCCAACTTCAT-3′ and 5′-CAGCCATCTTATTCCTTTCC-3′ for c-fos, and primers 5′-GTCAAGGGATGGTATTTATG-3′ and 5′-TTCCAGCGTGT CTATGTT-3′ for SIRT1 were used. The primers for real-time PCR and RT-PCR analysis of miR-16 and U6 were from Ambion. The RT-PCR was performed as described previously [Yi et al., 2010]. The real-time PCR was performed using SYBR green reagents and analyzed by delta CT method. GAPDH (for HuR, COX-2, c-fos, and SIRT1) or U6 (for miRNA) served as control.

CONSTRUCTS AND REPORTER GENE ASSAYS

For the construction of vectors expressing pre-miR-16 and miR-30, primers 5′-cgcggatccgcg (*Bam*H I) TACTTAAAATCTCCTT-3′ and 5′-ccatcgatgg (*Cal* I) AAACTTGATGGCA-3′, and primers 5′-cgcggatccgcg (*Bam*H I) GCCACTTGCCTATTT and 5′-ccatcgatgg (*Cal* I) GCCCTACTACGCTTTT were used to amplify the pre-miR-16 and miR-30 and inserted into the pLoxhyTk-LT vector (generously provided by Dr. Yusheng Cong) between the *Cal* I and *Bam*H I sites. For the construction of vectors expressing miR-16 and miR-30 shRNAs, gatccccAGTGCCTTAGCAGCACGTAttcaagaga TACGTGCTGCTAAGGCACTttttta and agcttaaaaaAGTGCCTTAGCAGCACGT AtctcttgaaTACGTGCTGCTAAGGCACTggg were inserted into psuper.retro vector (generously provided by Dr. Xiaowei Zhang) between the *Bgl* II and *Hin*d III sites. For reporter gene assays, HuR mRNA fragments CR, A, B (positions 1025–1879), and C (positions 2011–2889) were amplified by RT-PCR using following primers,

primers 5′-tcatctaga ATGGCCGAAGACTGCAGGGGTGAC-3′ and 5′-tcatctagaACGGGACCTGCCT GGAAAAGGA-3′ for CR, primers 5′-tcatctagaCCCGTTGCCACCTCCTGCT CAC-3′ and 5′-tcatctaga CCTTCCTCCGGGCTCCTGGTTTA-3′ for A, primers 5′-tcatctagaGT-TTGGCTTTGTGACC-3′ and 5′-tcatctagaGCCTGGAGCTTAGA TC-3′ for B, and primers 5′-tcatctagaGAGGCGTAAAATGGC-3′ and 5′-tcatctagaCAGGGAAAGGGGAG-3′ for C. To generate HuR fragment A1 mutating the miR-16 interaction site, the predicted miR-16 interaction sequence CGCCTGAAGATGTGTTGCTA within fragment A (positions 1881–1901) (available at website: http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl) was mutated as CGCCT-GAAGATTGCTCTGA by overlapping RT-PCR. The reporter vectors pGL3-CR, PGL3-A, PGL3-B, PGL3-C, or PGL3-A1 then was constructed by inserting the HuR mRNA fragments into the *Xba* I site of pGL3 vector (Promega, Madison, WI) and confirmed by sequence analysis. Transient transfection of HeLa cultures with the reporters was carried out by Lipofectamine 2000 (Invitrogen). Co-transfection of pRL-CMV served as an internal control. Firefly and renilla luciferase activities were measured with a double luciferase assay system (Promega) following the manufacturers' instructions. All firefly luciferase measurements were normalized to renilla luciferase measurements from the same sample.

PATIENT TISSUES

The patient tissues, including both tumor and adjacent normal tissues, were obtained and approved for use by the Third Hospital, Peking University Health Science Center. The samples were collected from September 2008 to January 2009.

PREPARATION OF PROTEIN AND RNA FROM IN VITRO CULTURES AND TISSUES

Total cellular protein and RNA were prepared as described previously [Yi et al., 2010]. The tissue protein and RNA were prepared from homogenized tumor and normal breast samples by RIPA buffer (for protein) and RNeasy Mini Kit (Qiagen, for RNA) following the manufacturer's protocol.

WESTERN BLOT ANALYSIS

For Western blot analysis, tissue or whole-cell lysates were size fractionated by SDS–PAGE and transferred onto poly-vinylidene difluoride (PVDF) membranes. Monoclonal antibodies recognizing HuR and GAPDH were from Santa Cruz Biotechnologies (Santa Cruz, CA). After secondary antibody incubation, signals were detected by SuperSignal WestPico Chemiluminescent Substrate (Pierce) following the manufacturer's instruction and quantitated by densitometric analysis with ImageMaster VDS software.

ANALYSIS OF NASCENT PROTEIN

One million cells were incubated with 1 mCi (1 Ci = 37 GBq) L-[^{35}S]methionine and L-[^{35}S]cysteine (Easy Tag EXPRESS, NEN/Perkin–Elmer) per 60-mm plate for 20 min, whereupon cells were lysed by using TSD lysis buffer (50 mM Tris, pH 7.5/1% SDS/5 mM DTT), and lysates were immunoprecipitated by using either monoclonal anti-HuR antibody (Santa Cruz Biotechnologies), anti-GAPDH antibody (Santa Cruz Biotechnologies), or IgG for 1 h at 4°C. After extensive washes in TNN buffer (50 mM Tris, pH 7.5/250 mM NaCl/5 mM EDTA/0.5% Nonidet P-40), immunoprecipitated material was resolved by 12% SDS–PAGE, transferred onto PVDF membranes, and visualized by using a PhosphorImager (Molecular Dynamics).

RESULTS

MIR-16 REPRESSES HUR TRANSLATION

The miRNA miR-16 and miR-125b were predicted to bind with the 3′UTR of HuR mRNA, as depicted in Figure 1A (schematic). The predicted interaction site of miR-16 with the 3′UTR of HuR by bioinformatics analysis locates between positions 1881 and 1901 (miRanda web server, available at: http://cbio.mskcc.org/cgi-bin/mirnaviewer/mirnaviewer.pl). To investigate the role of miR-16 in the regulation of HuR expression, human breast cancer MDA-MB-231 cells, previously used by Sengupta et al. [2003] for the study of HuR-mediated COX-2 mRNA decay, were transfected with a vector expressing miR-16 shRNA. Transfection of cells with a vector expressing miR-30, which was not predicted to bind with the HuR mRNA, or control shRNA was used as a negative control. Forty-eight hours later, whole-cell lysates were prepared for Western blot analysis to assess the protein level of HuR. As shown in Figure 1B, knockdown of miR-16 reduced miR-16 by ~84.9% on average (middle panel) and led to ~3.5-fold increase of HuR in protein level relative to that observed in control shRNA expressed cells (left panel). As a negative control, knockdown of miR-30 reduced miR-30 by ~79.3% on average (right panel) but had no effect on the level of HuR (left panel). To further assure the regulatory role of miR-16 on HuR expression, MDA-MB-231 cells were transfected with a vector expressing pre-miR-16 or pre-miR-30, the protein level of HuR was then assessed by Western blotting. As shown in Figure 1C, transfection of cells with vector expressing pre-miR-16 increased miR-16 by ~12.4-fold (middle panel) on average and reduced HuR protein level by ~70% (left panel), compared with that observed in empty vector transfected cells. As a negative control, transfection of cells with vector expressing pre-miR-30 increased miR-30 by ~10.4-fold (right panel) but had no effect on altering the protein level of HuR (left panel). Although miR-125b was also predicted to bind with the 3′UTR of HuR, as depicted in the right panel of Figure 1A, knockdown of miR-125b by transfecting cells with antisense miR-125b could not alter the protein level of HuR (Fig. 1D). Therefore, miR-16 may act as a negative regulator for HuR expression.

Thus far, microRNAs have been described as the regulators for the turnover or translation of target mRNAs. To further address the mechanism by which miR-16 regulates HuR, the total cellular RNA was prepared from cells described in Figure 1B,C and subjected to RT-PCR analysis. As shown in Figure 2A,B by RT-PCR and real-time PCR analyses, neither over-expression nor knockdown of miR-16 could significantly influence the mRNA levels of HuR, suggesting that miR-16 may regulate HuR expression at the level of translation. To confirm this point, MDA-MB-231 cells transiently expressing miR-16 shRNA were incubated with L-[^{35}S] methionine and L-[^{35}S] cysteine for 20 min, cell lysates were then prepared and the nascent HuR protein was analyzed by immunoprecipitation. As shown in Figure 2C, nascent HuR protein level in miR-16 shRNA expressed

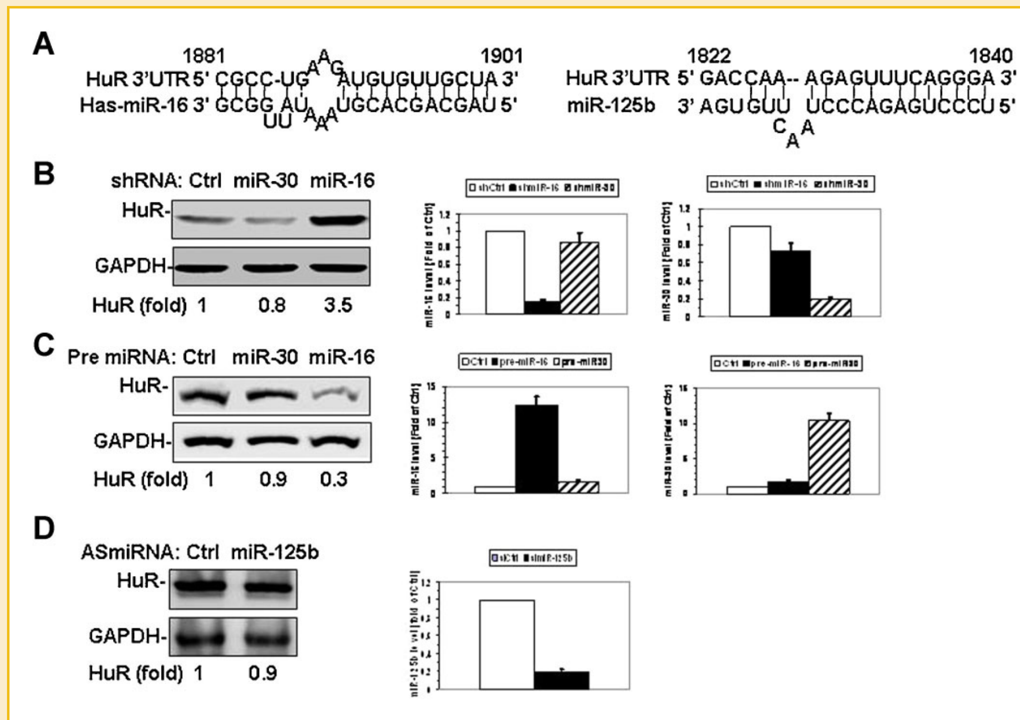

Fig. 1. miR-16 represses HuR expression. A: Schematic presentation depicting the predicted binding sites of miR-16 and miR-125b with HuR 3′UTR. MDA-MB-231 cells were transfected with vectors expressing shRNAs (B) or pre-miRNAs (C) of miR-16 or miR-30. Forty-eight hours later, total cell lysates or RNA was prepared for Western blot (left panel) or real-time PCR (middle and right panels) to analyze the protein levels of HuR or monitor the levels of miR-16 and miR-30. GAPDH served as a control. The Western blot data are representatives of three independent experiments. D: MDA-MB-231 cells were transfected with antisense miR-125b. Forty-eight hours later, total cell lysates or RNA was prepared for Western blot (left panels) or real-time PCR (right panels) to analyze the protein levels of HuR or monitor the levels of miR-125b, as described in (B) and (C).

cells was ~3.1-fold higher than that observed in empty vector transfected cells. As a negative control, knockdown of miR-16 did not substantially influence the levels of nascent GAPDH protein. These results suggest that miR-16 regulates HuR expression at the translational level.

HuR acts as an important stabilizer of mRNAs encoding cancer-related factors such as COX-2, c-fos, and SIRT1 [Wang et al., 2001; Sengupta et al., 2003; Abdelmohsen et al., 2008]. Therefore, we next asked whether the miR-16-HuR regulatory pathway is functional to the down-stream targets of HuR. To address this question, the mRNA levels of COX-2, c-fos, and SIRT1 in miR-16 silenced cells were tested by real-time PCR. As shown in Figure 2D, the mRNA levels of COX-2, c-fos, or SIRT1 in miR-16 silenced cells were ~8.7-, ~9.0- or ~9.8-fold higher on average than their levels in control shRNA expressed cells, while the mRNA levels of COX-2, c-fos, and SIRT1 only exhibited mild increase (~1.6-, ~2.0-, and ~1.9-fold, on average) in miR-30 silenced cells. These results suggested that miR-16 represses HuR translation, thereby inhibiting the mRNA levels of HuR targets.

INTERACTION OF MIR-16 WITH HUR 3′UTR AND THE ANALYSIS OF HUR 3′UTR HETEROLOGOUS REPORTER

Next, we investigated into the mechanism how miR-16 regulates HuR translation. For this purpose, biotinylated miR-16 and HuR transcripts [coding region (CR) and 3′UTR (A)] depicted in Figure 3A as well as RNA and cytoplasmic extracts of MDA-MB-231 cells were prepared and used for pull-down analysis as described in the Materials and Methods Section. Consistent with the prediction by the computer analyses (Fig. 1A), as shown in Figure 3B (upper panels), HuR mRNA could be detected by RT-PCR in miR-16, but not in control siRNA pull-down materials (Fig. 3B, upper panels); and miR-16 could be detected from the pull-down materials of the HuR 3′UTR (fragment A), but not from that of the HuR CR (Fig. 3B, bottom panels). As controls, GAPDH mRNA was undetectable in the pull-down materials of miR16, and U6 RNA was not detected in the pull-down materials of HuR transcripts.

To further test whether the association of miR-16 with the HuR 3′UTR was important for the regulation of HuR by miR-16, we constructed a series of pGL3-derived reporter constructs containing HuR fragments CR, A, B, C, or A1 [Fig. 3A,C(left), schematic]. The fragment A, but not fragments CR, B, and C, bears the predicted miR-16 interaction site (Fig. 1A). The fragment A1 is the mutant of fragment A mutated the miR-16 binding site (see the Materials and Methods Section). MDA-MB-231 cells were co-transfected with vector expressing miR-16 shRNA or the empty vectors plus pGL3, pGL3-CR, pGL3-A, pGL3-B, pGL3-C, or pGL3-A1 reporter vector along with pRL-CMV control reporter. Forty-eight hours later, firefly luciferase activity was determined and normalized against renilla luciferase activity. Total RNA was prepared for RT-PCR analysis to monitor the mRNA levels of luciferase, renilla luciferase, and GAPDH. As shown in Figure 3C (right), expression of miR-16

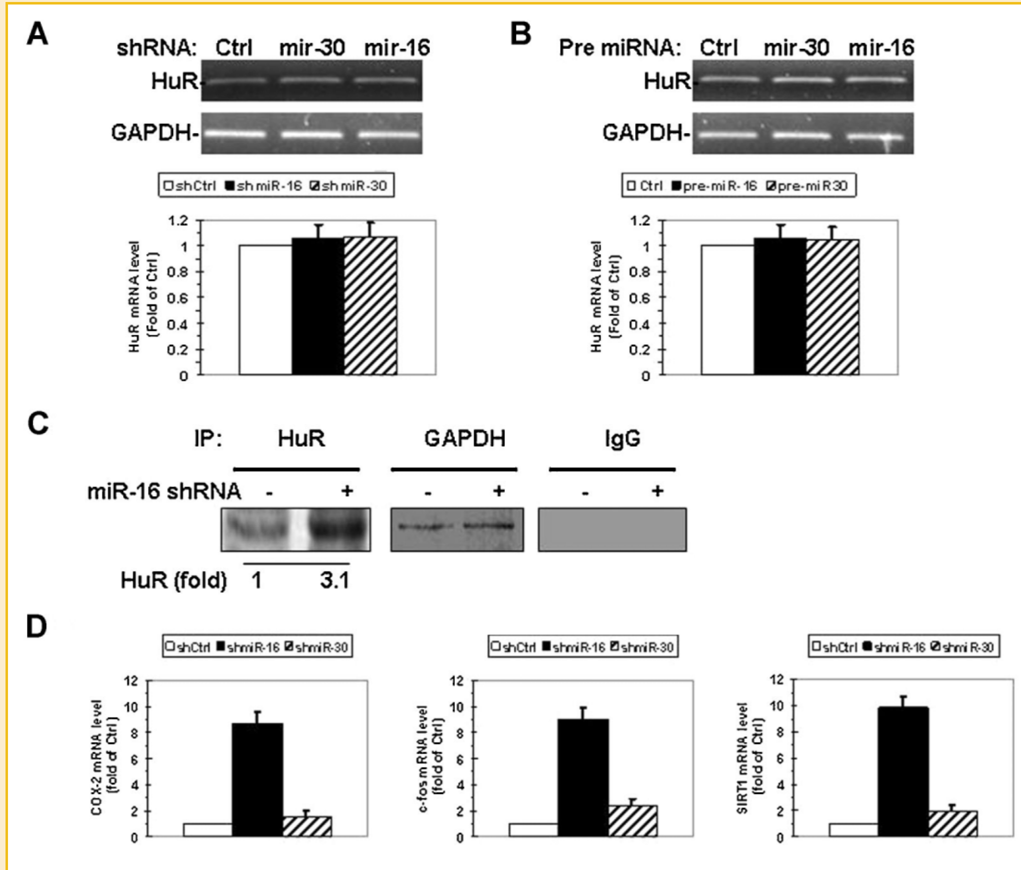

Fig. 2. miR-16 represses HuR translation and the expression of HuR target mRNAs. A,B: Total cellular RNA used in Figure 1B,C was subjected to RT-PCR (upper panels) and real-time PCR (bottom panels) analyses to assess the mRNA levels of HuR. The real-time PCR data represent the mean ± SE from five independent experiments. C: De novo synthesized HuR was analyzed in miR-16 shRNA expressed MDA-MB-231 cells, as described in the Materials and Methods Section. D: Total RNA prepared from cells described in Figure 1B and (A) of this figure was subjected to real-time PCR analysis to assess the mRNA levels of COX-2, c-fos, and SIRT1. Values represent the mean ± SE from five independent experiments.

shRNA greatly increased the luciferase activity of pGL3-A (~5.8-fold), but not that of PGL3, PGL3-CR, PGL3-B, PGL3-C, and PGL3-A1. In keeping with our finding that neither over-expression of pre-miR-16 nor knockdown of miR-16 could influence the levels of HuR mRNA (Fig. 2A,B), the mRNA levels of luciferase (Luci.), renilla luciferase (Reni.), and GAPDH of all the reporters described in Figure 3C were not altered by miR-16 knockdown (Fig. 3D). These results suggest that miR-16 interacts with HuR in the 3′UTR; and the miR-16 interaction site in the 3′UTR of HuR mediates the translational repression of HuR by miR-16.

REDUCTION OF MIR-16 IS ACCOMPANIED BY THE ELEVATION OF HUR IN HUMAN BREAST CANCER

The elevation of HuR has been linked to the high levels of COX-2 and other tumor-related factors in human carcinogenesis as well as to reduced survival in patients suffered from breast, ovarian, and gastric adenocarcinomas. To test the significance of miR-16 for the expression of HuR in human tumorigenesis, we collected tissue samples from 25 patients suffering from invasive ductal breast carcinoma (age range, 35–48). The adjacent normal breast tissue was used as a control for each of the tumor samples. Due to limited size of normal tissues, only 13 pairs of samples obtained sufficient protein (from normal tissue) for Western blotting. Of these samples, 11 pairs of samples obtained sufficient RNA for real-time PCR analysis. These protein or RNA samples then were subjected to Western blot to assess the protein levels of HuR or to real-time PCR to analyze the levels of miR-16 as well as the levels of HuR mRNA. As shown in Figure 4A, in the study population, 10 of 13 (77%) samples exhibited over ~3-fold increase of HuR protein level in tumor tissues, which was, on average, ~8.5-fold higher than that seen in normal tissues. As a control, the average protein level of GAPDH in tumor tissues was comparable to that observed from normal tissues (~0.93-fold vs. 1.00, on average) (Fig. 4B). In contrast to the tremendous increase of HuR protein in tumor tissues, only a moderate increase of HuR at mRNA level (~1.5-fold, on average) was observed (Fig. 4C), indicating that translational regulation may act as a major mechanism for the elevation of HuR in human breast cancer.

Next, we studied the correlation of miR-16 to HuR expression in human breast cancer. Because miR-16 was undetectable in both normal and tumor tissues of the sample number 6, only 10 pairs of samples was employed for the analysis of miR-16 by real-time PCR (Fig. 4D, upper panel). The results showed that the miR-16 levels in

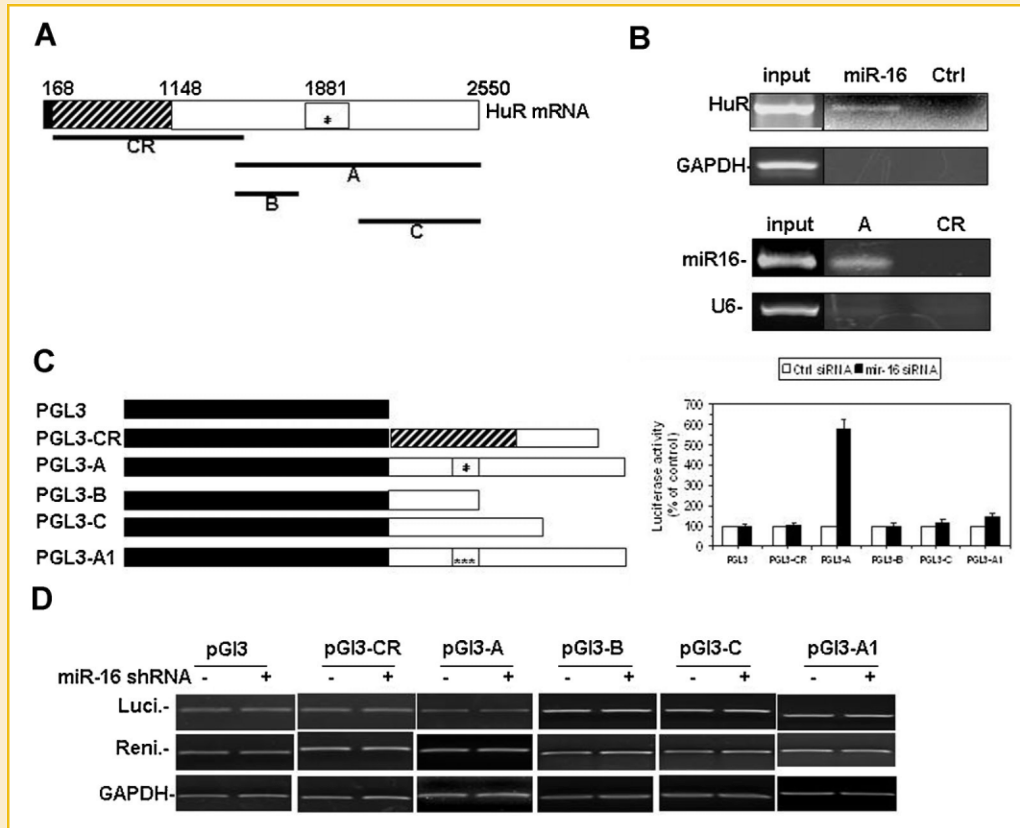

Fig. 3. Interaction of miR-16 with HuR 3′UTR and the analysis of HuR 3′UTR heterologous reporter. A: Schematic presentation of HuR transcripts derived from the coding region (CR) and 3′ UTR used in this study. B: Upper panels: RNA pull-down assays were performed using biotinylated miR-16 miRNA. RT-PCR of HuR and GAPDH from a 0.1 μg portion of total RNA, and RT-PCR of GAPDH mRNA (negative control) from the pull-down materials were included. Bottom panels: RNA pull-down assays were performed using biotinylated HuR mRNA fragments to detect bound miR-16 by RT-PCR. RT-PCR of miR-16 and U6 from a 5 μg portion of whole-cell lysates (Lys.), binding U6 (negative control) to HuR mRNA were included. C: Left: Schematic presentation of PGL3 reporters derived from the coding region (CR) and various 3′ UTR fragments of HuR used in this study. Right: MDA-MB-231 cells were co-transfected with a vector expressing miR-16 or control shRNA plus pGL3, pGL3-CR, pGL3-A, pGL3-B, pGL3-C, or pGL3-A1 reporter vector along with pRL-CMV control reporter. Forty-eight hours later, firefly luciferase activity was determined and normalized against renilla luciferase activity. Values represent the mean ± SD from five independent experiments. D: RNA prepared from cells described in (C) was subjected to RT-PCR to analyze the mRNA levels of luciferase, renilla luciferase, and GAPDH.

tumor tissues, on average, were ~73% lower than that observed from normal tissues, while the relative levels of HuR translation (protein/mRNA ratio) in tumor tissues were ~11.5-fold higher than that observed from normal tissues (Fig. 4D, bottom panel). Seven pair of the samples (70%, samples 1, 2, 9, 10, 16, 20, and 21) exhibited reverse correlation between HuR and miR-16 (reduced miR-16 but increased relative HuR translation) (Fig. 4D, upper and middle panels). Two pair of the samples (20%, samples 8 and 11) exhibited nearly unchanged miR-16 and mild change of relative levels of HuR translation. Thus, these results suggest that a decrease in miR-16 may exist in association with an increase in the level of HuR protein in a substantial proportion of human breast cancers.

DISCUSSION

The present study provides novel insight into the regulation of HuR in human breast cancer. Intervention of miR-16 level in MDA-MB-231 cells by transfecting a vector express pre-miR-16 reduced HuR expression, while knockdown of miR-16 by a shRNA approach elevated HuR expression. The regulation of HuR by miR-16 is basically an event at translational level because (1) neither over-expression of pre-miR-16 nor knockdown of miR-16 could alter the mRNA levels of HuR (Fig. 2A,B); (2) the nascent HuR protein is induced by miR16 silencing (Fig. 2C); and (3) knockdown of miR-16 markedly increases the luciferase activity of pGL3-A without altering its mRNA levels (Fig. 3C,D). Investigation into the mechanism underlying showed that miR-16 could interact with HuR mRNA at the 3′UTR (Fig. 3B). Interaction with HuR mRNA is a necessary step for the regulatory role of miR-16, since reporters that do not contain the miR-16 interaction motif (pGL3-CR, pGL3-B, and pGL3-C) or contain the mutated miR-16 interaction motif (pGL3-A1) cannot respond to miR-16 silencing (Fig. 3C).

The elevation of HuR is of great importance for the pivotal role of HuR in human breast cancer [Dixon et al., 2001; Erkinheimo et al., 2003; Denkert et al., 2004a,b; Suswam et al., 2005]. Because only moderately increase of mRNA was detected (Fig. 4C), regulation at

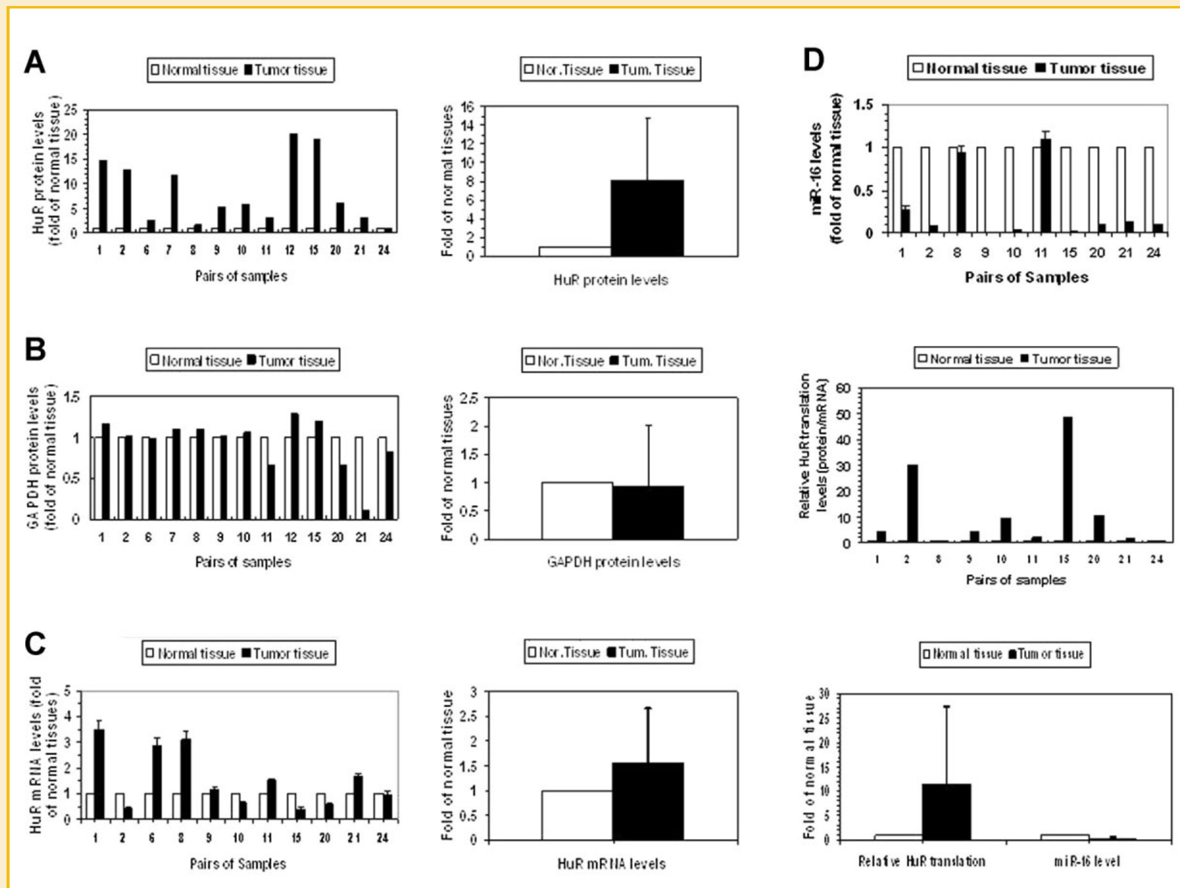

Fig. 4. miR-16 is inversely correlated to the expression of HuR in human breast cancer. Left: Protein extracts prepared from normal and tumor tissues were subjected to Western blot analysis to assess the expression of HuR (A) and GAPDH (B), respectively. The results from each pair of samples were quantified and represented as fold of normal tissue. A,B: Right: The results from left panel were represented as mean $\pm$ SD. C: RNA prepared from normal and tumor tissues was subjected to real-time PCR to assess the levels of HuR mRNA; the results were represented as fold of normal tissues from three independent experiments (left). Right: The results from the left panel were represented as mean $\pm$ SD. D: Upper: RNA samples used in (C) was subjected to real-time PCR to assess the levels of miR-16 (fold of normal tissues). Middle: The relative HuR translation (protein/mRNA ratio) was included. Bottom: The results from the upper and middle panels of (D) were represented as mean $\pm$ SD.

levels other than transcription and mRNA turnover may be more important for the up-regulation of HuR in human breast cancer. The miR-16-HuR regulatory pathway may be critical for the elevation of HuR in human breast cancer. The supporting evidence is that the increased HuR protein or HuR protein/mRNA ratio (relative increase of HuR translation) is accompanied with reduced expression of miR-16 in human breast tissues (Fig. 4A,D). Apart from miR-16, the translational regulation of HuR by microRNAs such as miR-519 and miR-125a has been reported recently [Abdelmohsen et al., 2008; Guo et al., 2009]. However, over-expression or knockdown of these microRNAs always exhibits mild influences on the expression of HuR (~2- to 3-fold). Therefore, it is plausible to postulate that other regulatory factors (e.g., other microRNAs, RBPs) may also involve in the regulation of HuR. These factors may cooperatively or independently regulate HuR and eventually lead to a complex consequence, to elevate the expression of HuR in human cancer. For example, recent studies have revealed a positive feed back regulation of HuR either through alterative mRNA turnover [Wijdan et al., 2009] or nuclear export [Yi et al., 2010]. It is possible that the elevation of HuR may be initiated by the reduction of above microRNAs and further strengthened through the positive feedback mechanism in the progression of human cancers.

In respect to the expression of tumor-related genes, miR-16 was reported to repress the expression of COX-2, TNF-α, bcl-2, MCL1, CCND1, and WNT3A at post-transcriptional level [Jing et al., 2005; Cimmino et al., 2006]. Interestingly, down-regulation of miR-16 occurs in chronic lymphocytic lymphoma (CLL), pituitary adenomas, small cell lung cancer, leukemia, and prostate carcinoma [Bottoni et al., 2005; Bonci et al., 2008; Calin et al., 2008; Bandi et al., 2009]. The current study described that HuR is another target of miR-16 in human breast cancer. Given that COX-2, TNF-α, and bcl-2 mRNAs are important targets of HuR in cancers, miR-16 may regulate the stabilization of these mRNAs through two pathways. First, miR-16 may directly destabilize their transcripts. Second, it may destabilize these target mRNAs through repressing the translation of HuR.

In human cancers, where clearly more genes are abnormally expressed than mutations, it is plausible that conditions associated with cancer also arise as a consequence of deregulated gene expression rather than mutation. Therefore, knowledge of the

mechanism serving to regulate gene expression is likely to aid our understanding of cancer. Because miR-16 and other microRNAs may act as global regulators for various tumor-related genes, strategies targeting these microRNAs in cancer may prove to be a more successful approach.

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