HuR and the bioenergetic signature of breast cancer: a low tumor expression of the RNA-binding protein predicts a higher risk of disease recurrence

Álvaro D. Ortega1,2, Sandra Sala1, Enrique Espinosa1, Manuel González-Barón3 and José M. Cuezva1,2,*

1Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid. 2CIBER de Enfermedades Raras and 3Servicio de Oncología Médica, Hospital Universitario La Paz, Universidad Autónoma de Madrid, 28049 Madrid, Spain

*To whom correspondence should be addressed. Tel: +34 911 964 618; Fax: +34 911 964 420. Email: jmceuzeva@cbm.uam.es

Downregulation of the catalytic subunit of the mitochondrial H+–ATP synthase (β-F1-ATPase) is a hallmark of many types of cancer. The expression of β-F1-ATPase is stringently controlled by posttranscriptional mechanisms. Herein, we pursue the identification of β-F1-ATPase messenger RNA-binding proteins (β-mRNABPs) that interact and could define the bioenergetic phenotype of the cancer cell in order to establish its relevance as markers of breast cancer progression. RNA immunoprecipitation and RNA affinity chromatography identify HuR as a β-mRNA-binding protein that interacts with the 3′-untranslated region of the transcript. Subcellular fractionation and high-resolution immunoelectron microscopy revealed the cofractionation and presence of HuR in subcellular structures associated to liver mitochondria. Analysis of the expression level of HuR in a cohort of breast carcinomas shows its association with the degree of alteration of the bioenergetic phenotype of the tumor. Moreover, HuR expression is shown to be an independent marker of breast cancer prognosis. A low tumor expression of HuR predicts a higher risk of disease recurrence in early stage breast cancer patients as assessed by clinical and bioenergetic markers of prognosis, strongly supporting the incorporation of HuR as an additional marker for the follow-up of these patients. Mechanistically, overexpression experiments and short hairpin RNA-mediated silencing of HuR in human embryonic kidney and HeLa cells indicate that HuR is not regulating β-F1-ATPase expression. Overall, the participation of additional RNA-binding proteins in controlling β-F1-ATPase expression and therefore in defining the bioenergetic signature of the cancer cell is expected.

Introduction

The metabolic reprogramming of cancer is nowadays established at both the molecular and functional levels. A conserved feature describing the energetic phenotype of most human tumors when compared with paired non-tumor tissues is that the expression of the catalytic subunit of the H+–ATP synthase (β-F1-ATPase) is significantly reduced and linked to a concurrent increase in the expression of markers of the glycolytic pathway (1–4). The β-F1-ATPase is the catalytic subunit of the H+–ATP synthase and thus a bottleneck in the provision of metabolic energy by mitochondrial oxidative phosphorylation. The proteomic index that relates markers of both energy-generation pathways define a “bioenergetic signature” that quantitatively informs of the rates of glucose utilization by the cell or tissue (5). In addition, the bioenergetic signature of colon (1), lung (5,6) and breast (3) tumors provides relevant markers of cancer prognosis. Moreover, it has been suggested that the metabolic phenotype of the cancer cell, as revealed by its bioenergetic signature, could afford a predictive marker of the cellular response to chemotherapy and a new target for cancer therapy (7–9).

Several interpretations have been put forward to explain the aerobic glycolysis of the cancer cell [2] and references therein]. More recently, we have suggested that the shift in the energetic phenotype of tumors illustrates the re-entrance of the cells of that particular tissue into the cell cycle because proliferation is incompatible with the bioenergetic activity of mitochondria and requires of the biosynthetic precursors derived from glycolytic intermediates (5,10–12). However, the specific molecular mechanisms that trigger the downregulation of the mitochondrial β-F1-ATPase in the cancer cell still remain to be determined (2). In this regard, the expression of β-F1-ATPase during development of the liver (13–16), progression through the cell cycle (11) and in liver carcinogenesis (2,17) has been shown to be regulated by posttranscriptional mechanisms that affect the subcellular localization, the stability and the translation of the messenger RNA (mRNA). The 3′-untranslated region (3′UTR) of β-F1-ATPase mRNA is essential for efficient translation of the transcript (13,18) due to its activity as an internal ribosome entry site (19). It appears that translation masking of the mRNA is due to the binding of β-F1-ATPase mRNA binding protein (β-mRNABPs) to the 3′UTR of the transcript that presumably hamper its essential role in translation (2,13,17).

The AU-rich element-binding protein HuR is a central regulator of posttranscriptional gene expression (20). The activity and subcellular localization of HuR are tightly regulated during the cell cycle (21,22) and HuR in turn regulates the expression of cell cycle relevant proteins such as cyclins A and B1 (22) and p21 (23). Cellular stress (24,25) and/or phosphorylation (21) promote the translocation of HuR to the cytoplasm where it can bind and stabilize target mRNAs. Moreover, HuR expression is increased in most human cancers (26) and its increased cytoplasmic localization has been related to a poor outcome in breast (27), ovarian (28,29) and gastric (30) cancer patients. Several mRNAs that code for mitochondrial proteins have been identified as HuR-interacting mRNAs in a high throughput screening (31). More recently, a study has documented the interaction of HuR with the AU-rich element of the 3′UTR of β-mRNA (32). Because of the pivotal role played by HuR in posttranscriptional regulation of gene expression, in this study we have addressed the following: (i) the possible interaction of HuR with β-mRNA at both the molecular and cellular levels; (ii) its functional role in posttranscriptional control of β-F1-ATPase expression and (iii) the association of HuR expression with the bioenergetic phenotype of tumors in a cohort of eighty-nine breast cancer patients (3). The results showed that HuR is an independent marker of breast cancer prognosis that identifies patients at high risk of recurrence in the early stages of the disease.

Materials and methods

Tissue specimens and clinical information of patients

Frozen tissue sections obtained from surgical specimens of eighty-nine patients who had an operation for invasive breast carcinoma at the Hospital Universitario La Paz between 1991 and 2000 were used (3) (see supplementary material Online). Patients’ medical records were reviewed, and identifiers coded to protect patient confidentiality. The Institutional Review Board approved the project. Patients who received chemotherapy or radiotherapy before surgery were excluded from the study. Supplementary Table S1 (available at Carcinogenesis Online) provides a summary of the clinical characteristics of the cohort of patients as well as of the expression level of HuR in each clinical group. For more details see ref. (3).

Abbreviations: BEC index, bioenergetic cellular index [beta/Hsp60]/GAPDH; β-F1-ATPase, β-subunit of the mitochondrial H+-ATP synthase; β-mRNABPs, β-F1-ATPase messenger RNA-binding proteins; β-mRNA, β-F1-ATPase mRNA; β-RNP, β-F1-ATPase ribonucleoprotein; CP, coat protein; DFS, disease-free survival; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HNK, human embryonic kidney; hRNA, hybrid RNA; IP, immunoprecipitate; MBP, maltose-binding protein; mRNA, messenger RNA; PBS, phosphate-buffered saline; shRNA, short hairpin RNA; 3′UTR, 3′-untranslated region.

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Affinity purification of HuR

Cell cultures

Human embryonic kidney (HEK) 293, hepatocarcinoma (HePG2) and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. Normal rat kidney cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum. Cells were cultured at 37°C and 7.5% CO₂ up to ~80% confluence. Cells were scrapped off from the plates after two phosphate-buffered saline (PBS) washes.

Immunoprecipitation of RNA

Cellular extracts (~10⁶ cells) were prepared after cross-linking of the cells with 1% formaldehyde in PBS for 10 min at room temperature with gentle shaking. After, the cells were incubated with 0.25 M glycerine in PBS for 5 min at room temperature. Cellular pellets were resuspended in radioimmuno precipitation assay (RIPA) buffer (0.25 M NaCl, 1% Triton X-100, 0.1% sodium dodecil sulfate, 0.5% NaDOC in 50 mM Tris–HCl pH 8.0) supplemented with protease inhibitors, sonicated (four 15 s pulses at maximum power) and left on ice for 5 min. Extracts were centrifuged at 12,000 g for 15 min at 4°C and the resulting supernatant incubated for 1.5 h at 4°C with protein A or G sepharose precoated with specific antibodies. The antibodies used for RNA immunoprecipitation were anti-HuR (Santa Cruz Biotechnology, Santa Cruz, CA), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Abcam, Cambridge, UK) and anti-AKAP149 (BD Biosciences, San Jose, CA). After formation of the immune complexes, the resin was washed three times with RIPA and three times with High stringency RIPA buffer (1 M NaCl, 1% Triton X-100, 0.1% sodium dodecil sulfate, 0.5% NaDOC, 1 M urea, 1 mM ethylenediaminetetraacetic acid in 50 mM Tris–HCl, pH 8.0). The revision of the chemical cross-linking and elution of the immunoprecipitated material was carried out by incubating in a buffer containing 5 mM ethylenediaminetetraacetic acid, 10 mM dithiothreitol, 1% sodium dodecil sulfate and 50 mM Tris–HCl pH 8 for 45 min at 70°C. The RNA was extracted from the samples using the RNeasy kit (QiAgen, Hilden, Germany) and precipitated using Glycolide as a carrier (Ambion, Austin, TX). For complementary DNA synthesis, 1 µg of RNA (input) or all the RNA immunoprecipitated were used as templates using 2.5 µM of random hexamers, 1 µL of RNase inhibitor and 2.5 µL of MULV reverse transcriptase (Roche Applied Science, Mannheim, Germany). Polymerase chain reaction (PCR) amplification reactions were carried out using as template 1/10 of the volume of the complementary DNA and the following primers—for β-F1-ATPase, hF1-forward: cacacggttgcaggtg and hF1-reverse: tcaatgtgcccacca; for HMGCR, HMGCR-forward: gaccaacccatatactgctt and HMGCR-reverse: ttgaaagtgctttctctgtaccc. For quantitative polymerase chain reaction, we used 1/50 of the volume of the complementary DNA as a template and the same primers set for β-F1-ATPase and the primers GAPDH-forward: agcagacagttagtggcgg and GAPDH-reverse: gcagcatgcaagcaatagc for GAPDH. Quantification was performed as described in supplementary material (available at Carcinogenesis Online).

Affinity purification of HuR

For the purification of RNA-binding proteins, we have adapted a previously described affinity chromatography method (33). Cellular extracts were prepared after washing the cells with RLN buffer (140 mM NaCl, 1.5 mM MgCl₂, 1 mM dithiothreitol in 50 mM phosphate buffer, pH 7.4). The recovered cells were counted before lysis (20 million cells/ml) in RLN-T buffer (RLN buffer supplemented with 0.5% Triton X-100 and the cocktail of protease inhibitors ethylenediaminetetraacetic acid free Roche Applied Science, Mannheim, Germany) for 5 min in ice. Nuclei and cell debris were removed by centrifugation (5 min at 800g) at 4°C. The post-nuclear extract was freeze–thawed three times and centrifuged for 15 min at 12,000g. Glycerol was then added to a final concentration of 5% and the samples were stored at ~70°C until used. The hybrid RNAs (hRNAs) used as baits were produced by in vitro transcription (T7 MEGAscript kit, Ambion) of plasmids that contained the human β-mRNA full-length, β3 sequence (16) or 3'UTR of β-mRNA (18,19) upstream the DNA coding for three plasmid MS2 coat protein (CP)-binding sites (33). Protein extracts (~12–16 mg in 2 ml) were incubated with 0.1 nmol of each hRNA for 1 h at 4°C. After 1 nmol of purified maltose-binding protein (MBP)-MS2 CP was added and incubated for an additional hour. The complexes were recovered on 0.2 ml of amylase (New England Biolabs, Ipswich, MA). The resin was extensively washed with RLN buffer before elution of RNABPs with RNase A (10 µg/ml) and maltose (100 µM). In a set of experiments, cellular extracts were ultraviolet cross-linked to the hRNA (16) before the addition of the fusion MBP-MS2 CP. In these experiments, the resin was sequentially washed with RLN buffer (twice), RLN supplemented with 0.3 M NaCl (one) and RLN supplemented with 1% Nonidet P-40 before the elution with RNase and maltose. Proteins were precipitated from the different fractions and blotted with antibodies against HuR and polyA binding protein as above indicated.

Cellular transfections and reporter assays

For HuR overexpression experiments, 3 × 10⁵ cells were seeded 24 h prior transfection. Three micrograms of plasmids containing HuR (pCMV-Sport6), β-galactosidase (kindly provided by Dr Kosik) or pcDNA (mock) were transfectioned using Plus and Lipofectamine reagents (Invitrogen, Carlsbad, CA). Cells were harvested 24–48 h after transfection. For green fluorescent protein (GFP) reporter experiments, plasmids containing GFP with the 3'UTR of β-mRNA (GFP-3'β) (11) were cotransfectioned at 1/10 with regard to HuR or pcDNA plasmids. After transfection, cells were recovered and processed for western blot and flow cytometry. A total of 20,000 events were analyzed by flow cytometry in a FACScalibur (BD Biosciences, San Jose, CA) and data analysis was carried out with Cell Quest software for Mac.

HuR knock-down experiments

A total of 8 × 10⁴ cells were seeded in 12-well plates 24 h prior transfection. One microgram of the plasmids encoding HuR short hairpin RNA (shRNA) (ID T352935) and non-efficient GFP shRNA (used as a control) (ID TR30003) were transfected as described above. Plasmids were purchased from OriGene (Rockville, MD). Gene silencing was assessed at 72 h posttransfection by western blot analysis.

In vitro determination of the rates of protein synthesis

Twenty-four hours after transfection of HEK cells with HuR or β-gal plasmids, the cells were subjected to metabolic labeling followed by immunoprecipitation. In brief, 70% confluent cells were incubated for 20 min in a Met- and Cys-depleted culture medium followed by 30 min incubation in a culture medium containing 130 µCl/ml ³²S-Met/Cys Premix (GE Healthcare, Uppsala, Sweden). The cells were then scraped, washed twice with cold PBS and processed for immunoprecipitation as described in Materials and Methods. The antibodies used for immunoprecipitation were rabbit polyclonal anti-β-F1-ATPase (1) and mouse monoclonal anti-Hsp60 (SPA-807, Stressgen, Victoria, Canada). The immunoprecipitates (IPs) were fractionated on sodium dodecil sulfate–polyacrylamide gel electrophoresis and processed for fluorography. The relative rate of β-F1-ATPase synthesis was determined by normalizing the incorporation of the radioactive tracer into the β-F1-ATPase protein relative to that incorporated into the mitochondrial HSP60 used as control. The incorporation of radioactivity into total trichloroacetic acid precipitable proteins was also determined (14).

Statistical analysis

Distribution of molecular markers was compared by Student’s t-test. Pearson’s coefficients were used for linear correlation studies. To determine the association between the expression levels of HuR with disease-free survival (DFS), a cut point value of 0.7 was used to define ‘high’ and ‘low’ expression groups. This cutoff point was the mean value of HuR expression in breast tumors that reveal a low deviation from the normal bioenergetic signature (see low medium signature in ref. 3). DFS was defined as the interval between the date of surgery and the date of tumor recurrence. Survival curves were derived from Kaplan–Meier estimates and compared by log-rank test. Cox proportional hazards regression methods were used for multivariate DFS analysis. Hazard ratios are presented with their 95% confidence intervals. Statistical tests were two sided at the 5% level of significance.

Results

HuR is a β-mRNA-binding protein

Exponentially growing HEK (Figure 1A) and HepG2 (Figure 1B) cells were processed for immunoprecipitation with antibodies against several RNABPs in order to assess the putative interaction of these proteins with β-mRNA. The IPs were subsequently analyzed by polymerase chain reaction for the presence of β-mRNA (Figure 1A and B) and other non-related RNAs used as controls. Western blots of the IPs (Figure 1A, upper panel) illustrated that the antibodies IP their respective antigens with different efficiencies. RNA immunoprecipitation coupled to reverse transcription–quantitative polymerase chain reaction analysis illustrates that β-mRNA was enriched in the IP of HuR when compared with input or the IPs obtained with antibodies against other RNABPs (GAPDH and AKAP149) (Figure 1A, lower histogram) that could be considered herein as non-specific immunoglobulins of the immunoprecipitation assays. The β-mRNA was also amplified in HuR IPs when using other human (HepG2) (Figure 1B) and rat (normal rat kidney) cell lines (data not shown). The lack of amplification of other cellular mRNAs (GAPDH in Figure 1A and...
HuR interacts with human β-mRNA (A and B) Extracts from human HEK (A) and HepG2 (B) cells were immunoprecipitated with antibodies against different RNA-binding proteins (AKAP149, GAPDH and HuR) and the corresponding protein identified by western blot in the IPs (upper panel in A). RNA from the IPs and from the starting material (input) was extracted and β-mRNA detected by quantitative polymerase chain reaction (lower histogram in A). HMGCR mRNA was also amplified to illustrate the specificity of the RNA–protein interaction (B). (C and D) HuR was purified using a tagged β-mRNA affinity chromatography method that is based on the utilization of a hRNA built by the MS2 CP-binding sites (in gray) and the RNA targets of interest (in black) for binding of the corresponding RNPAs (RNA-binding proteins, circles). The RNA was then bound by a fusion protein (castle shape form) made of the MS2 CP (door of castle) and the MBP (tower of castle) so that the RNA–protein complex formed could be anchored on an amylose column (gray-inverted crown). The proteins bound to hRNA were specifically eluted by digestion of the RNA used as bait with RNase A. In (D), the affinity-purified proteins were subjected to western blot for the detection of HuR and poly A binding protein 1, the latter an abundant cellular RNAb used as a control of the specificity of the purification procedure. Φ and β3, respectively, indicate the absence of hRNA and presence of β3 hRNA in the purification scheme. β3 is a sequence region of β-mRNA that does not interact with cellular proteins (16). 3′ UTR and β-FL identify purifications in which hRNA contained the 3′ UTR and the full-length β-mRNA.

HMGCR in Figure 1B) suggested that HuR specifically interacts with β-mRNA within the cellular context.

HuR interacts in vitro with the 3′ UTR of β-mRNA
The interaction between β-mRNA and HuR was further confirmed in in vitro assays. For this purpose, we developed chimERIC RNAs using sequences of β-mRNA and the RNA hairpins that bind the CP of phage MS2 with high affinity (hRNA in Figure 1C), following a previously described strategy (33). The developed RNAs consisted on the full-length β-mRNA, the regulatory 3′ UTR of β-mRNA (3′ UTR) and a region of the open reading frame of β-mRNA (β3) that does not interact with cellular proteins (16). The hRNAs were incubated with extracts derived from HEK cells and the putative RNA–protein complexes formed were subsequently retained on amylose columns by means of a fusion protein (MBP-CP) that contained the MBP (able to bind amylose) and the MS2 CP (able to bind the RNA hairpins) (Figure 1C). The RNA-binding proteins (in Figure 1C) were specifically eluted from the column by digestion of the hRNA with RNase A (Figure 1C).

Figure 1D shows the specific detection of HuR in the eluted proteins when the hRNA used as bait contained the 3′ UTR of β-mRNA. The specificity of this interaction is illustrated by the lack of immunoactivity when the purification scheme omitted the inclusion of a hRNA (Φ in Figure 1D) or the RNA included as bait does not interact with cellular proteins (β3 in Figure 1D). Moreover, an antibody against another abundant cellular RNAbp such as poly A binding protein did not reacted with the eluted proteins (Figure 1D) indicating the specific interaction of HuR with the 3′ UTR of β-mRNA. It is interesting to note that the amount of HuR eluted from the column when the full-length β-mRNA was used as bait was much less than that when using the 3′ UTR of β-mRNA (Figure 1D), suggesting that secondary structures of the RNA could partially mask the HuR-binding element in the 3′ UTR of the transcript. Overall, these results indicate that HuR binds the human 3′ UTR of β-mRNA in agreement with a recent report (32).

HuR interacts, cofractionates and colocalizes with β-F1-ATPase ribonucleoprotein complex
In rat liver, β-mRNA is contained in a large β-F1-ATPase ribonucleoprotein (β-RNP) complex attached to the outer mitochondrial membrane (34,35). The specificity of the interaction between HuR and the 3′ UTR of β-mRNA was further shown in normal rat kidney cells (Figure 2A). In these studies, the 3′ UTR was covalently attached to its interacting proteins by ultraviolet radiation before proceeding to purification (36). Figure 2A shows that HuR is retained on the column after high-stringent washing conditions (see W2 and W3 in Figure 2A) and is eluted only after RNase A digestion of the hRNA used as bait (see RNase in Figure 2A). These results strongly suggest that HuR and the 3′ UTR of β-mRNA were specifically interacting at the time of ultraviolet irradiation.

In rat liver, the β-RNP complex is a large round-shaped structure of ~150 nm in diameter that cofractionates with mitochondria and polysomes on sucrose gradients at a high density (16). Consistent with the reported interaction between HuR and β-mRNA (Figures 1 and 2A), we found that a fraction of HuR cofractionated with β-mRNA (Figure 2B) and mitochondria as revealed by the partitioning of β-F1-ATPase protein in gradient fractions (Figure 2B) and polysomes (data not shown). However, HuR also localized to other fractions of the gradient suggesting its involvement in the formation of additional RNP complexes (37,38).

High-resolution immunoelectron microscopy of human liver thin sections (Figure 2C) and of the gradient fractions containing partially purified β-RNP complexes (Figure 2D) allowed the identification of HuR in the vicinity of mitochondria (see gold particles in Figure 2C) and in electron-dense round-shaped structures attached to mitochondria (see gold particles in Figure 2D) (16,34), strongly suggesting the involvement of HuR in the localized posttranscriptional expression of mammalian β-mRNA.

HuR and the bioenergetic signature of breast cancer
The interaction of HuR with the 3′ UTR of β-mRNA, an essential regulatory element required for efficient translation of the transcript (13,18,19), suggested that HuR might be controlling the expression of the mitochondrial protein and thus primarily responsible for promoting the altered bioenergetic signature that characterizes most neoplasia syndromes (1,4) and specifically that of breast cancer (3). For this reason, we first determined the expression of HuR in parallel with the markers of the bioenergetic signature in normal and tumor biopsies obtained from the same patients operated from breast ductal invasive adenocarcinomas (Figure 3A). Remarkably, we observed that the expression of HuR is barely detectable in normal samples, whereas its expression is highly increased in the tumors (Figure 3A). Next, we...
studied the relationship of HuR with the bioenergetic phenotype of breast cancer in a retrospective cohort of 89 breast carcinomas previously characterized from the point of view of the bioenergetic signature and for which the clinical and follow-up information of the patients was available (3). Hierarchical clustering of breast carcinomas by the expression level of markers of the bioenergetic signature (3) generated three different groups of carcinomas establishing the low, medium and highly divergent phenotypes from the normal breast bioenergetic phenotype (see Figures 1 and 3 in ref. 3). Interestingly, HuR expression correlated with the bioenergetic low, medium and high signature of the carcinomas (P < 0.001, χ² test). Consistent with this finding, the expression level of HuR discriminated two different groups of carcinomas with significantly different expression levels of the relevant markers (β-F1-ATPase, Hsp60 and GAPDH) of the bioenergetic signature (Figure 3B). A high expression of HuR was associated with a high deviation from the normal breast bioenergetic phenotype, i.e. a drop in β-F1-ATPase concurrent with increased Hsp60 and GAPDH expression (Figure 3B and see ref. 3). In contrast to these findings, it is of interest to note that HuR expression did not correlate with clinical–pathological markers relevant for tumor progression such as nodal affectionation (P = 0.225, χ² test), clinical stage (P = 0.063, χ² test) and histological grade (P = 0.487, χ² test).

HuR is an independent marker of breast cancer progression

Univariate analysis showed that lymph node involvement (P = 0.001), stage disease (P = 0.001) and grade of differentiation (P = 0.041) were the clinical–pathological variables significantly associated with progression of the disease. Similarly, Kaplan–Meier survival analysis revealed that a low tumor expression of HuR predicted a worse prognosis for breast cancer patients (Figure 3C). Interestingly, it was observed that the 5 year distant DFS in low-risk patients, as assessed both by the clinical–pathological markers and by the bioenergetic signature of the tumor, was significantly reduced if HuR expression was low (Table I). The same happened in high-risk patients (β-cluster C2 in Table I and see ref. 3), although there were very few patients in this last subgroup. These results were confirmed by individual expression data using an unsupervised hierarchical clustering presentation of HuR and the mitochondrial (β/Hsp60) and cellular (BEC) bioenergetic signatures (Figure 3D). This type of analysis discriminated the patients into two main groups: HB for high- and LB for low-bioenergetic signature, respectively (Figure 3D). Within each of these two groups, the expression level of HuR defines two additional subgroups: one with low (a in Figure 3D) the other with high (b in Figure 3D) HuR expression. The analysis shows that patients bearing tumors with a high-bioenergetic signature but with a low expression of HuR (HB-a in Figure 3D) are the ones who developed metastatic disease (red asterisk in Figure 3D), Kaplan–Meier DFS analysis (data not shown) confirmed (P = 0.002) the risk differences between the HB-a and HB-b subgroups. Multivariate Cox regression survival analysis using the clinical–pathological and bioenergetic markers previously shown to predict disease progression revealed that a low expression of HuR is an independent marker of prognosis (supplementary Table S2 is available at Carcinogenesis Online). Each multivariate analysis performed included three molecular markers and one clinical variable (see C, D and E in supplementary Table S2 available at Carcinogenesis Online) because nodal status, clinical stage and histological grade are not independent variables (see A in supplementary Table S2 available at Carcinogenesis Online).

HuR does not influence the posttranscriptional expression of β-F1-ATPase

The overexpression of HuR in HEK (Figure 4A) and HeLa cells (supplementary Figure S1A is available at Carcinogenesis Online) neither affect the relative expression of β-F1-ATPase protein (Figure 4A and supplementary Figure S1A is available at Carcinogenesis Online) nor significantly altered the cellular abundance of its mRNA (Figure 4B). Moreover, analysis of GFP expression driven from a
reporter that contains the 3’UTR of β-mRNA (GFP-3’β) (11), both by western blot (Figure 4C, histogram to the right) and by FACS analysis (Figure 4C, histogram to the left), revealed that HuR overexpression did not significantly affect GFP expression. Similar findings were obtained in HeLa cells (supplementary Figure S1 is available at Carcinogenesis Online). Furthermore, we observed that the in vivo relative rate of β-F1-ATPase synthesis in cells overexpressing HuR was not significantly different from that observed in control cells (Figure 4D, histogram to the right). However, it should be noted that HuR overexpression diminished the global rates of cellular protein

Fig. 3. HuR is overexpressed in breast cancer and provides a marker of tumor progression. (A) Protein samples from normal (N) and tumor (T) breast biopsies were taken from the same patient (B1–B3) and analyzed by western blot for the total expression levels of HuR and of markers of the bioenergetic signature (β-F1-ATPase, Hsp60 and GAPDH). A representative example obtained with three patients is shown. (B) Association of HuR expression levels with the markers of the bioenergetic signature in 89 primary breast ductal invasive adenocarcinomas (3). Relative protein expression levels were calculated as indicated under western blots in supplementary material (available at Carcinogenesis Online). Low and high HuR expression groups were defined as indicated in Materials and Methods. The mean ± SEM expression values of the markers in each group and P-values for these comparisons by Student’s t-test are indicated. A low total HuR expression in the carcinoma significantly correlates with a low deviation of the bioenergetic signature of the carcinomas as assessed by the expression levels of β-F1-ATPase, Hsp60 and GAPDH. (C) Kaplan–Meier DFS analysis for 89 breast cancer patients stratified by the expression level of HuR in the carcinoma. The plot shows a significant association of low HuR expression levels with a poor outcome for the patients. The log-rank test P-value is shown. (D) Unsupervised hierarchical clustering analysis (http://ep.ebi.ac.uk/EP/EPCLUST using Euclidean distances and average linkage method) of HuR and the mitochondrial (β/Hsp60) and cellular (BEC) bioenergetic signature in 89 breast tumor biopsies. Rows, samples; columns, markers. Color scale: red, high; black, normal and green, low. The dendrogram represents overall similarities in the expression profiles providing two clusters differing mainly on the bioenergetic signature (HB and LB, for high and low signature, respectively). Red asterisks identify the patients who developed metastasis. Note that in the HB group (patients bearing tumors with a low alteration of the bioenergetic signature, i.e. in the early stages of the disease) those patients with a low expression level of HuR (a subgroup) are the ones who develop metastasis.
synthesis when compared with controls (Figure 4D, histogram to the left), consistent with the relevant role of HuR in posttranscriptional gene expression (39). In agreement with these findings, knock-down of HuR by means of shRNA did not affect β-F1-ATPase expression in human cells (Figure 5A and 5B and supplementary Figure S1A is available at Carcinogenesis Online). Moreover, HuR silencing did not influence the expression of the GFP-3 β chimera (Figure 5C and supplementary Figure S1B is available at Carcinogenesis Online).

Overall, these results indicate that HuR is not controlling the expression of β-F1-ATPase and that posttranscriptional regulation of the bioenergetic signature of the cell is very complex being stringently controlled by additional players of the β-RNP (16).

Discussion

The RNA-binding protein HuR has been shown to play a pivotal epigenetic role in posttranscriptional regulation of its targeted genes. This is specially relevant in situations where the cellular abundance of the protein is increased, such as in human cancers (26) and under the stress conditions that operate in cancer cells (20,21,25,37). In this work, we show that HuR specifically binds the 3′ UTR of human and rat β-mRNA and that it colocalizes with the large β-RNP associated with mitochondria. Since the subcellular localization (16,35), the stability (14,17) and translation (2,11,13,15,17) of β-mRNA are tightly regulated in a variety of cellular processes including development, oncogenesis and the cell cycle, it would initially appear that HuR represents a key candidate for regulating the expression of β-F1-ATPase and thus in defining the altered bioenergetic phenotype of the cancer cell (1–6). Indeed, we do find that the expression level of HuR in breast tumors correlates with the alteration of the bioenergetic signature, adding further information to clinical-pathological and molecular markers of prognosis.

The 3′ UTR of β-mRNA is a key controlling element of the translation of the transcript that displays both in vitro (13) and in vivo (18) a translational enhancer activity because it is endowed with internal ribosome entry site activity (19), i.e. it is able to interact and recruit the translational machinery. The repression of β-mRNA translation in fetal rat liver (13) and in hepatomas (17) is triggered by the binding of regulatory proteins to the 3′ UTR of the mRNA interfering in this way with its role in translation (2,13). In these situations, the bioenergetic function of mitochondria is compromised (2,13,17), as it has been observed in most human cancers (1,3–5). The identification of HuR as a 3′ UTR β-mRNA-binding protein, in agreement with a recent report (32), raised a first relevant question regarding the actual role of HuR in posttranscriptional regulation of β-F1-ATPase expression in the cancer cell. We show that silencing of HuR in two different cell lines did not affect the expression level of β-F1-ATPase or of GFP driven from the GFP-3 β reporter (Figure 5 and supplementary Figure S1 is available at Carcinogenesis Online). In addition, a large over-expression of HuR, as it is observed in human tumors [(26) and see Figure 3], does not affect the expression of β-F1-ATPase at both the protein and mRNA levels (see Figure 4 and supplementary Figure S1 is available at Carcinogenesis Online). Moreover, no significant changes in the in vivo rates of synthesis of β-F1-ATPase, as assessed by the incorporation of 35S-methionine (see Figure 4D) and with reporter assays (see Figure 4C and supplementary Figure S1B is available at Carcinogenesis Online), were observed in cells overexpressing HuR. These results strongly argue against the possibility that HuR could inhibit β-mRNA translation by binding the 3′ UTR of β-mRNA interfering in this way with its role in translation (2,11,13,15,17).

Although the mechanism of participation of HuR in controlling the expression of β-F1-ATPase, and thus in defining the bioenergetic phenotype of the cancer cell, remains to be determined, our findings do not preclude that the broad-spectrum RNABP could be supporting other events for the correct posttranscriptional expression of this gene. The mitochondrial localization of HuR could suggest its participation in the sorting and/or anchoring of β-mRNA at its site of translation. In agreement with this suggestion, the subcellular localization of HuR is tightly regulated during the cell cycle (21,22) and in response to stress (20,25,37). Moreover, HuR could participate as scaffold for the assembly of the β-RNP, requiring the participation of additional proteins to define the metabolic fate of the transcript (38). In fact, it appears that the relevant β-mRNA-binding proteins involved in translation masking of β-mRNA (13,17) have a molecular mass >50 kDa and poly A-binding activity (41). Whatever the situation, the challenge ahead in the scenario of the β-RNABPs that control the expression of β-mRNA is the development and implementation of further genetic and/or proteomic approaches to identify the components of the β-RNP (38).

The function of some HuR-targeted genes support a role for the RNABP in proliferation (21–23,40). Consistent with this belief, HuR is overexpressed in most human cancers (26) representing a potential marker of cancer prognosis (27–29). These studies have evaluated HuR expression by immunohistochemistry and found that an...
increased cytoplasmic immunostaining of the protein predicts a worse prognosis for the patients (27–29). Recently, immunohistochemistry has been questioned for the evaluation of the expression of markers of cancer prognosis (43). We have determined the total cellular expression level of HuR in breast tumors by western blot and confirmed, in agreement with previous works by immunohistochemistry (27–29), that HuR expression is a marker of cancer prognosis. However, our results indicate that a low total expression of HuR in the tumor predicts a worse prognosis for breast cancer patients (Table I), which could be explained by the role of HuR as enhancer of the translation of the tumor suppressor p53 (39,44). Interestingly, a similar situation has been described for the RNABPs G3BP and insulin-like growth factor 2 mRNA-binding protein 1 that are of relevance in human cancer (45,46). Although these proteins have been reported to be overexpressed in different types of carcinomas, certain tumor cell populations that acquire the metastatic phenotype show a reduced expression of the RNABPs (46). Consistent with this idea, we suggest that breast tumors with a low expression of HuR are identifying the patients in the early stages of the disease that could develop metastasis [Figure 3C and Table I and supplementary Table S2 (available at Carcinogenesis Online)].

The metabolic reprogramming seems to be a necessary step required to progress during the cell cycle (11) and a response of the cancer cell to survive and to progress into malignancy (8,47). The bioenergetic phenotype of the cell could be assessed by a proteomic index (BEC index) that informs of the relative activity of the two pathways involved in cellular energetic metabolism (1). The BEC index has recently been shown to correlate both in vivo and in vitro with the rates of glucose capture and utilization by aerobic glycolysis in human carcinomas (5). Further, the BEC index is highly sensitive for the discrimination of normal and tumor biopsies (3,6) and a valuable tool for the prognosis of colon (1), lung (5,6) and breast (3) cancer patients, as well as to predict the response to chemotherapy in colon (7) and liver (8) cancer cells. Although the mechanistic aspects that relate HuR and the bioenergetic phenotype still require further investigations, it is clear from this study that HuR should be used as an additional marker for the prognosis of breast cancer patients. Indeed, we show that HuR and the markers of the bioenergetic signature are independent predictors of breast cancer outcome. Moreover, they are also independent markers of well-established clinical–pathological markers of breast cancer prognosis contributing to determine those patients with a high risk of recurrence when in the early stages of their disease. Overall, these findings strongly support the inclusion of HuR as an additional proteomic marker in the future translational development of clinical tests for the bioenergetic signature of breast cancer.

Supplementary material

Supplementary Tables S1 and S2 and Figure S1 can be found at http://carcin.oxfordjournals.org/.

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**Conflict of Interest Statement:** None declared.

**Fig. 5.** Silencing of HuR does not affect β-F1-ATPase expression. HEK cells were transfected with a 29 mer shRNA against HuR mRNA or a non-efficient shRNA used as control (Cont). (A) HuR knock-down was assessed in three different experiments by western blot using two different protein loads (1 and 1/5) of the control cells for comparison. Hsp60 and β-F1-ATPase expression were also determined in the same gels. The histograms show the expression level of each protein in control (sh-Cont) and HuR (sh-HuR) knock-down cells. The results are the mean ± SEM of four independent experiments. (C) HuR silencing as in (A) does not promote significant changes in the expression of GFP from a reporter bearing the 3′ UTR of β-mRNA (GFP-3′ β). The GFP-3′ β vector was cotransfected together with shRNAs at a 1/10 molar ratio. GFP expression was determined by western blot. The results were normalized versus control cells (sh-Cont) and are the mean ± SEM of three independent experiments.

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**References**


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