Published in final edited form as:

J Pathol. 2011 August ; 224(4): 529–539. doi:10.1002/path.2889.

Role of RNA binding protein HuR in ductal carcinoma in situ of the breast

Mira Heinonen^{1,2}, Annabrita Hemmes^{1,2}, Kaisa Salmenkivi¹, Kotb Abdelmohsen³, Suvi-Tuuli Vilén⁴, Marko Laakso^{2,5}, Marjut Leidenius⁶, Tuula Salo⁷, Sampsa Hautaniemi^{2,5}, Myriam Gorospe³, Päivi Heikkilä¹, Caj Haglund^{1,8}, and Ari Ristimäki^{1,2,*}

¹Department of Pathology, HUSLAB and Haartman Institute, Helsinki University Central Hospital and University of Helsinki, Finland ²Genome-Scale Biology, Research Program Unit, University of Helsinki, Finland ³LMBI, NIA-IRP, National Institutes of Health, Baltimore, MD, USA ⁴Institute of Dentistry, Biomedicum Helsinki, University of Helsinki, Finland ⁵Computational Systems Laboratory and Institute of Biomedicine, Biomedicum Helsinki, University of Helsinki, Finland ⁶Breast Surgery Unit, Helsinki University Central Hospital and University of Helsinki, Finland ⁷Department of Diagnostics and Oral, University of Oulu, Finland ⁸Department of Surgery, Helsinki University Central Hospital and University of Helsinki, Finland

Abstract

HuR is a ubiquitously expressed RNA-binding protein that modulates gene expression at the post-transcriptional level. It is predominantly nuclear, but can shuttle between the nucleus and the cytoplasm. While in the cytoplasm HuR can stabilize its target transcripts, many of which encode proteins involved in carcinogenesis. While cytoplasmic HuR expression is a marker of reduced survival in breast cancer, its role in precursor lesions of malignant diseases is unclear. To address this we explored HuR expression in atypical ductal hyperplasia (ADH) and in ductal in situ carcinomas (DCIS). We show that cytoplasmic HuR expression is elevated in both ADH and DCIS when compared to normal controls, and that this expression associated with high grade, progesterone receptor negativity and microinvasion and/or tumour-positive sentinel nodes of the DCIS. To study the mechanisms of HuR in breast carcinogenesis, HuR expression was silenced in an immortalized breast epithelial cell line (184B5Me), which led to reduction in anchorage-independent growth, increased programmed cell death and inhibition of invasion. In addition, we identified two novel target transcripts (CTGF and RAB31) that are regulated by HuR and that bind HuR protein in this cell line. Our results show that HuR is aberrantly expressed at early stages of breast carcinogenesis and that its inhibition can lead to suppression of this process.

Introduction

HuR is an mRNA-binding protein and a member of the Hu/ELAV-family ^{1, 2}. The other family members, HuB/HeIN1, HuC and HuD, are primarily found in the neuronal tissues, while HuR is expressed ubiquitously. HuR protein localizes predominantly to the nucleus, but it shuttles between the nucleus and the cytoplasm; in the cytoplasm HuR can stabilize its target transcripts and/or regulate their translation ^{3–5}. Aberrant expression of HuR associates with reduced survival in several types of adenocarcinomas in the gastrointestinal tract, ovaries and breast ^{6–11}. In addition, cytoplasmic HuR expression has been associated with

tamoxifen resistance in MCF7 breast cancer cells and inhibition of its expression increased breast cancer cell responsiveness to tamoxifen¹². However, it is not known whether HuR is expressed in precursor lesions of malignant conditions. Intraductal neoplastic lesions of the breast with an increased risk of invasive ductal breast cancer are atypical ductal hyperplasia (ADH) and ductal in situ carcinoma (DCIS)¹³. We have now explored HuR expression in ADH and DCIS specimens and its association with the clinicopathological parameters in DCIS. The mechanism of HuR in breast carcinogenesis was studied in an immortalized breast epithelial cell line, 184B5Me, by modulation of HuR expression and by measuring parameters relevant in carcinogenesis.

Materials and Methods

Patient samples

Altogether 76 DCIS patients who underwent sentinel node investigation between April 2001 and March 2005 at the Breast Surgery Unit of Helsinki University Central Hospital were included into the study (14; see also Supporting information, Supplementary methods). We were able to score immunoreactivity for HuR in 74 of the 76 cases (97%), since in two cases there was no representative tissue left. The median age at the time of the diagnosis was 56 (range 38–91) years. In addition, specimens from 71 women with ADH treated at the Helsinki University Central Hospital during 2006–2010 were included into the study. Median age at the time of the diagnosis was 55 (range 26–88) years. Finally, 66 breast tissue specimens from reduction mammoplasty surgery and without any histological alterations and operated at the Helsinki University Central Hospital during 2000–2010 were included in the study, representing a subgroup of healthy controls [median age 47 (range 18–75) years]. The study was approved by the Ethical Committee of the Helsinki University Central Hospital and the National Authority for Medicolegal Affairs (TEO 226/E6/06).

Immunohistochemistry

DCIS specimens were stained with HuR 19F12 and 3A2 antibodies. 19F12 antibody was used at a dilution of 1 : 10 000 (1 µg/ml; a kind gift from Dr Furneaux, UCHC, Farmington, USA) and 3A2 antibody at a dilution of 1 : 1000 (0.2 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The immunostaining protocol for HuR antibodies was carried out as described previously by Erkinheimo et al⁶, except that for the 19F12 antibody Tris–HCl, pH 8.5, buffer was used as an antigen retrieval buffer instead of Na–citrate. ADH and healthy control specimens were stained with HuR 19F12 antibody as described above.

HuR immunoreactivity was scored as described previously (8; see also Supporting information, Supplementary methods). Statistical analyses were done based on the HuR cytoplasmic positivity versus negativity. Immunostaining for MIB-1, oestrogen receptor (ER) and progesterone receptors (PR) had been assessed previously¹⁴.

Cell cultures

184B5Me human mammary epithelial cell line (a kind gift from Dr Stampfer, University of California, Berkeley, CA, USA) was obtained by transfection of immortalized 184B5 cells with a construct encoding for erbB2 oncoprotein and by selection for anchorage-independent colonies (15; see also Supporting information, Supplementary methods). 184B5Me cells were cultured in MCDB 170 growth medium (US Biological, Swampscott, USA) supplemented with 5 ng/ml epidermal growth factor, 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 70 µg/ml bovine pituitary extract, 5 µg/ml transferrin, 10 µM isoproterenol, 50 µg/ml amphotericin B and 50 µg/ml gentamicin (all reagents from Sigma-Aldrich, St. Louis, MO, USA). MCF7 and 293 FT cells were cultured in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine and antibiotics. The cells were cultured at 37 °C and 5% CO₂.

HuR silencing constructs, virus particle production and cell infections

To silence HuR expression, a lentiviral HuR shRNA construct was developed. pDSL_hpUGIH lentivector with target oligos were used. HuR shRNA target oligos were as follows: forward 5'-GATCCGCATGACCCAGGATGAGTTATTCAAGAGATAAATCATCCTGGGT CATGTTTTTTGGAAA-3', reverse 5'-AGCTTTTCCA AAAAACATGACCCAGGATGAGTTATCTCTTGAATAAATCATCCTGGGTCATGCG-3'. The control shRNA construct expressed a non-targeted, scrambled sequence of HuR shRNA oligo, and the oligos were as follows: forward 5'-GATCCGAACTTCGCGATGGATACATTCAAGAGATGTATCCATCGCGAAGTTCTT TTTTGGAAA-3', reverse 5'-AGCTTTTCCAAAAA GAACTTCGCGATGGATACATCTCTTGAATGTATCCATCGCGAAGTTTCG-3'. These oligos were planned with an Applied Biosystems siRNA Converter [www.ambion.com/techlib/misc/psilencer_converter.html].

Lentiviral particles were produced transfecting 293 FT cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) containing a mixture of three plasmids; target vector, packaging plasmid and a VSVg-coding envelope vector, according to the manufacturer's instructions. Transfected 293 FT cells were incubated at 37 °C and 5% CO₂ for 72 h, after which the supernatant was collected, sterile-filtered and used for infections.

To infect 184B5Me cells, the spin-down method was used. Briefly, cells were incubated with virus particle media containing 8 µg/ml polybrene for 10 min at 37 °C and 5% CO₂, centrifuged (Multifuge 3 S-R, Sorvall/Heraeus) at 2500 rpm for 30 min at room temperature and incubated again at 37 °C and 5% CO₂ for 2 h, after which the complete growth medium was changed on the cells. After cultivating the cells for 72 h, selection with hygromycin B (200 µg/ml; Invitrogen) was started. Lentiviruses were produced at the Biomedicum Virus Core Facility [www.biomedicumgenomics.fi/bvc].

Cell transfections

184B5Me cells were transfected with HuR siRNAs. HuR siRNA1 (siHuR1, 5'-AACATGACCCAGGATG AGTTA-3'; Dharmacon, Thermo Scientific, Lafayette, USA) sequence is the same as that used for the HuR shRNA construct and the second siRNA against HuR (siHuR2). ON-TARGET plus SMARTpool (Dharmacon, Thermo Scientific) is a siRNA pool containing four separate siRNA sequences against HuR, but not the siHuR1. siRNA ON-TARGET plus Non-targeting Pool (Dharmacon) was used as a control. The cells were treated with 100 nM siRNA, using Lipofectamine 2000 (twice the amount recommended in the manufacturer's instructions).

Protein extractions and western blot analysis

Total proteins were isolated using NucleoSpin® RNA/ protein kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Cytoplasmic and nuclear fractions were prepared from the cell cultures using a NE-PER nuclear and cytoplasmic extraction kit (Pierce Biotechnology, Rockford, USA). Primary antibodies used for immunoblotting were mouse monoclonal HuR 3A2 (1 : 2000), rabbit polyclonal HER-2 (1 : 1000; Upstate, Lake Placid, NY, USA), rabbit polyclonal ATF-2 (1 : 100; Santa Cruz Biotechnology), mouse monoclonal CTGF (1 : 50; Santa Cruz Biotechnology), rabbit polyclonal Rab31 (1 : 50; Santa Cruz Biotechnology), rabbit polyclonal anti-lamin A/C (1 : 500; Cell Signaling Technology, Dancers, USA), rabbit polyclonal anti-β-tubulin (1 : 500; Santa Cruz

Biotechnology) and goat polyclonal anti- β -actin (1 : 1000; Santa Cruz Biotechnology). Secondary antibodies used were goat anti-mouse (1 : 2000 for HuR, 1 : 500 for CTGF; Pierce Biotechnology), goat anti-rabbit (1 : 500; Pierce Biotechnology) and donkey anti-goat (1 : 2000; Santa Cruz Biotechnology).

Real-time reverse transcriptase–polymerase chain reaction (qRT–PCR)

RNA was isolated using a NucleoSpin® RNA/protein kit according to the manufacturer's instructions. RNA (1 μ g) was converted to cDNA and quantitative RT–PCR gene expression assays were performed using a GeneAmp 7500 sequence detection system according to the manufacturer's protocol (Applied Biosystems, Foster City, USA). TaqMan® gene expression array primers (Applied Biosystems) were used in this study and human 18S rRNA was used as an endogenous control. Data were analysed by the $2^{-\Delta\Delta C_t}$ method for comparing relative expression results.

Cell proliferation

Cell proliferation was measured using the fluorometric method CellTiter-Blue® Cell Viability Assay (Promega) according to the manufacturer's instructions. Cells were seeded on 96-well plates (1 $\times$ 10³ cells/well) and the amount of fluorescent signal was measured using a FluorStar Optima Microplate Reader (BMG Labtech, NC, USA).

Anchorage-independent growth

Cells (1 $\times$ 10⁴/ml) were suspended in 1 ml 0.25% agarose (GellyPhor, EuroClone Spa, Pero, Italy) layered over 1 ml 0.5% agarose base layer and supplemented with 2 ml complete culture medium in a six-well plate. After 10 days of culture, the cells were fixed with methanol and stained with Giemsa (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Cell colonies were photographed using a Leica MZFLIII microscope with $\times$ 3.2 enlargement and analysed with ImageJ Software.

Programmed cell death (anoikis)

Cells (5 $\times$ 10⁴/ml) were suspended in 3 ml 0.5% methylcellulose (Sigma-Aldrich) in complete growth medium and seeded onto bacterial plates (Greiner Bio-One GmbH, Frickenhausen, Germany). After 9 days of culture the cell colonies formed were counted.

Cell invasion

The ability of HuR-silenced cells to invade was studied using a myoma organotypic culture model¹⁶. Briefly, organotypic cultures were equilibrated in complete cell culture medium for 10 days at 4 °C with gentle rocking, and placed into Transwell inserts (Corning, USA). Cells (5 $\times$ 10⁵) were seeded on the tissue and allowed to attach overnight, after which the cultures were transferred onto uncoated nylon discs in 12-well plates in 1 ml culture medium. The cultures were maintained at 37 °C for 10 days and the cell culture medium was changed every 3 days. On day 10, organotypic cultures were fixed in 4% neutral buffered formalin overnight, dehydrated, bisected and embedded in paraffin. To identify invaded cells, pancytokeratin AE1/AE3 (1 : 150 dilution; Dako, Glostrup, Denmark) immunohistochemistry was carried out as previously described¹⁶. Invasion depth was determined by measuring the depth of the four deepest invaded cells and/or cell clusters per slide from four slides per sample.

Gene expression arrays

GeneChip® Human Genome U133 plus 2.0 (Affymetrix, Santa Clara, CA, USA) gene expression arrays were performed in duplicate at the Biomedicum Biochip Center [www.helsinki.fi/biochipcenter]. Gene expression arrays were analysed using CSC Chipster

v. 1.3.0 software for DNA Microarray data analysis [<http://nami.csc.fi>]. Microarray data were normalized using GeneChip Robust Multi-array Average (gcrma). The probes were re-annotated using alternative chip description file environments [hs133phsentrezg (hgu 133plus2)]. In the re-annotation process, ambiguous probes that map to more than one position in the genome were discarded, leaving 17 589 probe-sets for the analyses. Data were preprocessed filtering genes according to their standard deviations. Cut-off 95% for the data to be filtered out was used. A two-groups test was used for statistical analyses, p value cut-off 0.01.

mRNP immunoprecipitation for HuR

Endogenous RNA–protein complexes were immunoprecipitated (IP) from 184B5Me and MCF7 cell lysates, using 50% v/v suspension of Protein A-Sepharose beads (Sigma-Aldrich) precoated with 30 µg of either anti-HuR antibody (3A2) or IgG1 control (BD Pharmingen, Franklin Lakes, USA), as previously described by Lal et al¹⁷. mRNA levels of the ten target genes were studied using RT–qPCR. GAPDH was used as an endogenous control and the results were normalized to it. PTMA and EIF4EBP2 were positive controls.

Statistical analysis

The data were analysed using SPSS 16.0 for Mac (SPSS, Chicago, IL, USA). The association between HuR staining and clinicopathological parameters in DCIS was assessed using the χ^2 -test. Non-parametric Mann–Whitney U-test was used for statistical analysis to compare the groups in cell proliferation, anchorage-independent growth, anoikis and invasion assays (shown as mean $\pm$ SEM). $p < 0.05$ was considered statistically significant.

Results

HuR protein expression in breast specimens

Cytoplasmic HuR immunoreactivity was present in 29% (19/66) of the healthy breast tissue specimens, but appeared in a focal manner and localized to the luminal epithelial cells of the acinar and ductal structures (Figure 1A–C). In ADH samples, cytoplasmic HuR expression was found in 49% (35/71) and in DCIS in 47% (35/74) of the cases. In the ADH and DCIS specimens, the staining pattern was homogeneous and localized to the neoplastic epithelial cells (Figure 1D–G). Expression of HuR was found to be more frequent in the ADH ($p = 0.014$) and DCIS ($p = 0.025$) patients compared to the healthy controls. These results show that cytoplasmic HuR expression is elevated in preneoplastic lesions of invasive breast cancer.

Association of HuR expression with clinicopathological parameters in DCIS

The association of cytoplasmic HuR expression with clinicopathological parameters was studied in the DCIS specimens. With the 19F12 antibody we found cytoplasmic HuR expression to associate with high grade ($p = 0.021$) and negative PR status ($p = 0.026$; Table 1). Interestingly, HuR immunoreactivity also associated with a more aggressive form of the disease, when cases with microinvasion or tumour positive sentinel nodes were compared to cases with negative sentinel nodes and without invasion ($p = 0.012$). However, HuR expression did not associate with proliferation marker MIB-1 positivity ($p = 0.699$), which is consistent with our earlier results obtained from invasive breast cancer specimens (unpublished data). We confirmed these immunostaining results by using another HuR antibody (3A2), and were able to stain 73/74 19F12-stained specimens (in one case there was no representative tissue left). There was a strong association of immunoreactivity between the two HuR antibodies ($p < 0.001$; see Supporting information, Table S1). In addition, almost identical results were obtained with this alternative antibody (see

Supporting information, Table S1), i.e. association of cytoplasmic HuR expression with PR negativity ($p = 0.002$), high grade ($p < 0.001$) and either specimens containing microinvasion or cases with tumour-positive sentinel nodes ($p = 0.014$). In addition, ER negativity was found to associate with HuR immunoreactivity when using the 3A2 antibody ($p = 0.002$). These results are consistent with our previously published data on invasive breast cancer, in which cytoplasmic HuR expression is associated with an aggressive form of the disease and hormone receptor negativity^{8, 10}.

Modulation of HuR expression in breast epithelial cells

To explore the significance of HuR expression in breast epithelial cells, we chose to use 184B5Me cells (see also Supporting information, Figure S1). These cells model DCIS in the respect that they are clearly transformed in vitro, since they form colonies in anchorage-independent conditions but produce tumours at a very low frequency in vivo conditions (oral communication from Dr Stampfer; our own unpublished data; and¹⁸). We performed stable HuR silencing in 184B5Me cells by using lentiviral construct. Infection of 184B5Me cells with the shHuR construct led to a clear inhibition of HuR protein expression (Figure 2A). When nuclear and cytoplasmic fractions were studied separately, HuR expression was reduced in both the nuclear (Figure 2B) and the cytoplasmic (Figure 2C) fractions, showing near-complete inhibition of the cytoplasmic expression. This was an important observation, since cytoplasmic HuR is thought to be the functional pool of HuR with respect to mRNA stabilization and reflecting the carcinogenic properties of this protein¹⁹. At the mRNA level, HuR expression was reduced by 87% in HuR-silenced cells compared to controls (shHuR versus shSCR, $p = 0.017$; Figure 2D). Since HuR has been reported to regulate HER-2 expression in SKBR3 breast cancer cells²⁰, we investigated the expression of HER-2 in 184B5Me cells. While HER-2 protein and the transcript were readily detectable, modulation of HuR expression in these cells did not have an effect on HER-2 expression (see Supporting information, Figure S2).

Modulation of breast epithelial cell carcinogenic properties by HuR silencing

Growth of the 184B5Me cells was measured in two-dimensional (2D) cultures for 7 days. Inhibition of HuR expression with the shHuR construct led to a minor and transient inhibition of cell proliferation (Figure 3A). The ability of HuR-silenced cells to grow in anchorage-independent conditions was studied in soft agar. The number of colonies in soft agar was reduced by 44% in HuR-silenced cells compared to controls (mean 38 versus 68 colonies, respectively; $p < 0.001$; Figure 3B). In order to study a form of programmed cell death named anoikis, the HuR-silenced cells were grown in methylcellulose, in which they formed 45% fewer colonies compared to controls (mean 40.5 versus 73.3 colonies, respectively; $p = 0.050$; Figure 3C). Finally, invasion efficiency of the HuR-silenced cells was studied in an organotypic model¹⁶ and the mean invasion depth of the HuR-silenced cells was 730 (range 182–1318) μm , while it was 970 (range 418–1315) μm for the control cells ($p < 0.001$; Figure 3D). On average, the invasion efficiency of HuR-silenced cells was reduced by 25% (see Supporting information, Figure S3).

We also performed transient inhibition experiments of HuR by siRNAs and found that cell proliferation was not altered after HuR silencing (Figure 4A). However also here in the soft agar assay colony formation was reduced by 51% with HuR siRNA1 (colony counts, siHuR1 11.5 versus siCtr 23.3; $p < 0.001$) and by 42% with HuR siRNA2 (colony counts, siHuR2 13.6 versus siCtr 23.3; $p < 0.001$; Figure 4B).

Figure 4. Effect of transient HuR silencing was studied in 184B5Me cells transfected with two distinct HuR siRNAs or with the control siRNA (siCtr). (A) Effect in 2D proliferation

assay (n = 6). (B) Effect in soft agar colony-forming assay (n = 15). Results are shown as mean $\pm$ SEM

HuR-regulated transcripts

To investigate the effect of HuR silencing on gene expression in breast epithelial 184B5Me cells, we compared the control cells (shSCR) to the HuR-silenced (shHuR-treated) cells using gene expression arrays. We analysed the data using $p < 0.01$ as a cut-off. For over-expressed transcripts we selected only those that were induced by two-fold or higher and for under-expressed ones those that were 0.5-fold or lower. These cut-off values led to a gene list of 57 up-regulated mRNA species and 57 down-regulated ones (see Supporting information, Table S2). Endogenous HuR (ELAVL1) transcript, which itself is a HuR target²¹, showed 83.2% inhibition due to the shHuR treatment, and thus served as an important internal positive control for the down-regulated genes.

HuR binding motif rich in uracil has been identified from mRNAs⁴. Of the 57 down-regulated transcripts, 43 (75.4%) contained one or several putative HuR-binding motif(s) (see Supporting information, Table S2). Interestingly, up-regulated transcripts less frequently (29/54, 53.7%) contained the HuR-binding motif. These data suggest that the frequency of transcripts with this motif is higher in the pool of down-regulated transcripts. Since we were primarily interested in HuR-upregulated genes in breast carcinogenesis, we concentrated on transcripts whose expression was inhibited by shHuR, contained the putative HuR-binding motif⁴ and had previously been shown to play a role in breast carcinogenesis (based on PubMed search, using the terms 'breast cancer, and shortcut for the gene and/or full name of the gene'). This led us to a list of 10 genes (Table 2). Interestingly, in the top 10 down-regulated transcripts that contain HuR RNA-binding motif(s), eight, including HuR (ELAVL) itself, were found to be related to breast carcinogenesis (see Supporting information, Table S2). We verified the down-regulation of the 10 target genes by qRT-PCR and the amount of the inhibition varied from 35.8% to 86.3% (Table 2). Transient transfection with a HuR-targeted siRNA molecule that contained the same sequence as the previously described shRNA construct down-regulated eight of the 10 transcripts, of which seven were verified by using an alternative HuR siRNA sequence (Table 2). Finally, we validated the transcripts that bind HuR by using immunoprecipitation. These data show that HuR directly binds three of the ten target transcripts in 184B5Me and in a breast cancer cell line MCF7 (Figure 5A). Positive binding was found between HuR and activating transcription factor 2 (ATF2), connective tissue growth factor (CTGF) and a RAS oncogene family member, RAB31. The transcripts' fold enrichment in HuR immunoprecipitation varied from 4.11 to 11.90 when compared to the housekeeping gene GAPDH (Figure 5A). In addition, ATF2 and RAB31 protein expression levels were down-regulated after HuR silencing, as presented by immunoblotting (Figure 5B). Transient HuR silencing did not affect CTGF expression, although 55.8% reduction was achieved with stable HuR inhibition (Table 2). It is possible that CTGF requires long-term inhibition in order to be down-regulated. Additionally, we could not detect CTGF protein expression, which was probably due to low protein expression level in the cells. HuR-binding motifs are presented in Figure 5C.

Network analysis

To obtain a global view of alterations in the HuR-silenced cells, gene set network analyses were performed, using the computational platform Moksikaan²². Biological processes that were affected by HuR silencing in breast epithelial cells were epithelial cell differentiation, cell adhesion, nucleosome assembly, wounding, response to different stimulus, regulation of biological quality and phospholipid homeostasis (see Supporting information, HuR study, p. 33). KEGG pathway analysis showed that HuR silencing affected several cancer

development-related pathways, such as p53, MAPK and TGF β signaling pathways in breast epithelial cells (see Supporting information, HuR study, p. 18), and the expression of several genes related to cell transformation was altered after HuR silencing (see Supporting information, HuR study, pp. 37–67).

Discussion

We have shown that HuR is aberrantly expressed in precursor lesions of a malignant disease. Additionally, modulation of HuR expression was shown to suppress properties related to transformation in breast epithelial cells, and several HuR target transcripts were identified, two of which are novel HuR-binding transcripts. Cytoplasmic HuR expression is elevated to the same extent in ADH (49%) and DCIS (47%) compared to normal controls (29%). It is important to note that the pattern of expression in pre-invasive lesions (ADH and DCIS) was homogeneous, whereas in the normal controls it was of a focal nature. This may reflect the monoclonal nature of the neoplastic lesions, whereas in the normal breast tissue HuR may be under endo-, para- and/or autocrine control²³. The frequency of cytoplasmic HuR protein expression in the precursor lesions of invasive breast cancer are similar to those found in invasive breast cancer (30–75%)^{8, 10, 24, 25}, which indicates that HuR accumulates to the cytoplasm at an early stage of the carcinogenic process. Since cytoplasmic HuR is thought to be the functional portion of HuR by mediating mRNA stabilization or translation of cancer-promoting proteins¹⁹, our results support the hypothesis that HuR contributes to carcinogenesis of the breast.

Previous studies have reported tumour-positive sentinel node findings in up to 16% of patients with DCIS²⁶. We decided to include these patients in the study, since these may represent a very early stage of missed invasion in breast cancer. DCIS specimens were stained with two distinct HuR antibodies, exhibiting almost identical results, and this associated with high grade, hormone receptor negativity and a more aggressive form of the disease, i.e. micro-invasion and/or tumour-positive sentinel node finding. In invasive breast cancer studies, similar associations between elevated HuR expression and poorly differentiated tumours and hormone receptor negativity have been reported^{8, 10, 24, 25, 27}. It is thus important to investigate whether inhibition of HuR has an effect on breast carcinogenesis.

A tissue culture model representative of DCIS was used to study the role of HuR on transformation and gene expression¹⁵. Neither stable nor transient silencing of HuR expression altered proliferation of the 184B5Me cells, which suggests that in this in vitro model HuR does not mediate its carcinogenic properties by affecting proliferation. This is consistent with our clinical data, which show that the proliferation marker MIB-1 did not associate with HuR expression in the DCIS specimens. However, HuR silencing reduced anchorage-independent growth, suppressed invasive properties and increased programmed cell death (anoikis). HuR has previously been associated with many cellular processes, including differentiation and cellular responses to DNA-damaging stimuli^{28, 29}. Additionally, it has been shown to regulate the expression of many cancer-related genes¹⁹. Similarly to our results, Mazan-Mamczarz et al³⁰ have shown that modulation of HuR expression affected pathways involved in cellular transformation in MCF10A breast epithelial cells transformed with MCT-1 oncogene, and Woo et al³¹ have shown in breast cancer cells that HuR supports cellular motility and invasion by regulating c-fms. These results suggest that HuR participates in processes important in transformation, invasion and programmed cell death.

We also identified three target transcripts (ATF2, CTGF and RAB31) for HuR as shown by gene expression arrays, qRT-PCR, immunoblotting and HuR-binding assay in the 184B5Me

cells. Of these transcripts, CTGF and RAB31 are novel HuR targets, whereas ATF2 has previously been linked to HuR in intestinal epithelial cells, where polyamines were shown to mediate ATF2 regulation via HuR³². Interestingly, these transcripts have previously been associated with apoptosis, proliferation, migration and invasion in breast cancer^{33–35}. However since we did not study all of the modulated genes after HuR silencing, we cannot conclude that the main targets of HuR are any of these three factors alone. In fact, a more likely scenario is that functions such as transformation, programmed cell death and invasion are a sum of several factors acting in concert. This is supported by our gene network analyses and by the work of Mazan-Mamczarz et al.³⁶, who showed that in a breast cancer tissue culture model the enhanced tumorigenicity produced by MCT-1 oncogene caused alterations in the association of HuR with several of its target mRNAs.

Our results show that cytoplasmic HuR expression is aberrantly expressed in precursor lesions of invasive breast cancer, and silencing of HuR affects multiple properties of transformed breast epithelial cells. These results show a new role for HuR in the early stage of breast carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Päivi Peltokangas and Tuire Koski for excellent technical assistance, Johanna Partanen and Juha Klefström for help with the virus constructs, and Siina Junnila and Outi Monni for help with the Affymetrix array analysis. MH is a student in the Biomedical Helsinki Graduate School and ML in the Finnish Doctoral Programme in Computational Sciences. The authors are grateful for funding from Helsinki University Central Hospital Research Funds (to AR and CH), the Sigrid Juselius Foundation (to AR and CH), the Finnish Cancer Organization (to AR), the Finnish Cultural Foundation (to MH), the Paulo Foundation (to MH) and NIA-IRP of the National Institutes of Health, USA (to KA and MG).

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Figure 1.

Expression of HuR in healthy breast tissues and in precursor lesions of invasive breast cancer. Focal staining pattern of cytoplasmic HuR immunopositivity in healthy breast tissue specimens (A). Cytoplasmic HuR positivity in acinar structures (B) and in the luminal epithelial cells of a duct (C). Immunostaining results of HuR cytoplasm-negative (D) and -positive (E) ADH specimens, and cytoplasm-negative (F) and -positive (G) DCIS specimens. Original magnifications: (A) $\times 200$, (B–G) $\times 400$. Acini indicated by arrowhead in (A) are enlarged in (B); similarly, duct marked * is enlarged in (C)

Figure 2.

Expression of HuR was modulated using lentiviral constructs in a breast epithelial cell line (184B5Me). Down-regulation was obtained by expression of a small hairpin sequence for HuR (shHuR), which was controlled by a scrambled sequence (shSCR). Expression levels of HuR protein in total cell lysates (A), nuclear fraction (B) and cytoplasmic fraction (C) were detected by immunoblotting. Expression of the HuR transcript was measured by qRT-PCR (D). Results are shown as mean $\pm$ SEM (n = 9)

Figure 3.

Effect of HuR silencing on cell proliferation, anchorage-independent growth, programmed cell death (anoikis) and invasion efficiency of 184B5Me cells. (A) Effect of HuR silencing in 2D cultures. Black columns represent control cells (shSCR) and grey columns HuR-silenced cells (shHuR; $n = 18$). (B) Effect on anchorage-independent growth in three-dimensional (3D) colony-forming soft agar assays ($n = 16$). (C) Effect on anoikis in methylcellulose ($n = 9$). (D) Effect on invasion in an organotypic model ($n = 16$). Results are shown as means $\pm$ SEM

Figure 4.

Effect of transient HuR silencing was studied in 184B5Me cells transfected with two distinct HuR siRNAs or with the control siRNA (siCtrl). (A) Effect in 2D proliferation assay (n = 6). (B) Effect in soft agar colony-forming assay (n = 15). Results are shown as mean $\pm$ SEM

Figure 5.

HuR binding to the HuR-regulated transcripts were studied in 184B5Me breast epithelial and MCF7 breast cancer cells by immunoprecipitation (IP) and qRT-PCR. (A) HuR binding to target transcripts. Results are normalized to GAPDH, whereas PTMA and EIF4EBP2 served as positive controls for HuR binding. Results are shown as mean $\pm$ SEM (three independent experiments). (B) Protein expression levels of the HuR-bound transcripts were studied in HuR-silenced 184B5Me breast epithelial cells (shHuR) and their controls (shSCR). (C) Schematic picture presenting HuR-binding motifs in the 3' UTR of the target transcripts. Black rectangles illustrate HuR-binding motifs and the numbers above show the exact positions of the binding motifs in the sequence. Dark grey rectangle presents the 5' UTR; light grey the coding sequence (CDS); and the white part is the 3' UTR. Numbers below this rectangle describe the length of the mRNA and show the positions where 5' UTR changes to CDS and further to 3' UTR. 'gi' and NM numbers indicate NCBI reference sequences (www.ncbi.nlm.nih.gov/nucore/).

Table 1

Association of HuR protein expression with clinicopathological parameters in DCIS, as determined by 19F12 antibody

		<u>Cytoplasmic HuR expression</u>		<i>p</i> *
		Negative	Positive	
Grades	Low	25	13	0.021
	High	14	22	
ER	Negative	7	9	0.137
	Positive	28	15	
PR	Negative	13	16	0.026
	Positive	22	8	
MIB-1	Negative	4	2	0.699
	Positive	31	22	
Microinvasion and tumour-positive sentinel node	Negative	35	23	0.012
	Positive	4	12	

* χ^2 test.

Table 2

Ten further validated transcripts from the gene expression array that were down-regulated after HuR silencing in 184B5Me cells, contained putative HuR binding motif(s) and have previously been associated with breast carcinogenesis

Description	Number of putative HuR binding motifs*	Reduction in the array (%)	qRT-PCR inhibition (%)			HuR binding (IP)	Ref
			shHuR	siHuR1	SiHuR2		
<i>ATF2</i>	Activating transcription factor 2	59.9	35.8	40.8	21.6	Yes	32, 35
<i>CTGF</i>	Connective tissue growth factor	70.2	55.8	101	119	Yes	33
<i>DPYD</i>	Dihydropyrimidine dehydrogenase	93.9	86.3	BDL	BDL	No	37
<i>GRB14</i>	Growth factor receptor-bound protein 14	61.4	71.2	39.4	37.9	No	38
<i>MTUS1</i>	Mitochondrial tumour suppressor 1	55.7	49.8	15.0	10.5	No	39
<i>PRKD1</i>	Protein kinase D1	72.9	65.0	18.3	42.2	No	40
<i>RAB27A</i>	RAB27A, member of <i>RAS</i> oncogene family	72.3	62.2	58.9	39.6	No	41
<i>RAB31</i>	RAB31 member of <i>RAS</i> oncogene family	78.9	61.0	18.1	23.6	Yes	34
<i>TFF12</i>	Tissue factor pathway inhibitor 2	91.3	84.6	65.2	133	No	42
<i>VCAN</i>	Versican	84.4	83.3	46.5	3.4	No	43

BDL, below detection limit.

* Lopez de Silanes et al4.