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ORIGINAL ARTICLE

Regulation of non-AU-rich element containing c-fms proto-oncogene expression by HuR in breast cancer

H-H Woo[1], Y Zhou[1], X Yi[1], CL David[1], W Zheng[1], M Gilmore-Hebert[2], HM Kluger[3], EC Ulukus[4], T Baker[1], JB Stoffer[1] and SK Chambers[1]

[1]*Arizona Cancer Center, University of Arizona, Tucson, AZ, USA;* [2]*Department of Pathology, Yale University School of Medicine, New Haven, CT, USA;* [3]*Internal Medicine/Oncology, Yale University School of Medicine, New Haven, CT, USA and* [4]*Dokuz Eylul Universitesi, Izmir, Turkey*

The role of RNA-binding proteins in cancer biology is recognized increasingly. The nucleocytoplasmic shuttling and AU-rich RNA-binding protein HuR stabilizes several cancer-related target mRNAs. The proto-oncogene c-fms, whose 3′untranslated region (3′UTR) is not AU-rich, is associated with poor prognosis in breast cancer. Using a large breast-cancer tissue array ($N=670$), we found nuclear HuR expression to be associated with nodal metastasis and independently with poor survival ($P=0.03$, RR 1.45), as well as to be co-expressed with c-fms in the breast tumors ($P=0.0007$). We described c-fms mRNA as a direct target of HuR *in vivo*, and that HuR bound specifically to a 69-nt region containing 'CUU' motifs in 3′UTR c-fms RNA. Overexpressing or silencing HuR significantly up- or down-regulated c-fms RNA expression, respectively. We also found that known glucocorticoid stimulation of c-fms RNA and protein is largely dependent on the presence of HuR. HuR, by binding to the 69-nt wild type, but not mutant, c-fms sequence can regulate reporter gene expression post-transcriptionally. We are the first to describe that HuR can regulate gene expression by binding non-AU-rich sequences in 3′UTR c-fms RNA. Collectively, our findings suggest that HuR plays a supportive role for c-fms in breast cancer progression by binding a 69-nt element in its 3′UTR, thus regulating its expression.
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Introduction

HuR, a member of the Elav/Hu family of RNA-binding proteins, is an important regulator of post-transcriptional gene expression (Brennan and Steitz, 2001). Overexpression of HuR, a nucleocytoplasmic shuttling protein, increases the *in vivo* stability of many mRNAs, which contain AU-rich elements (ARE) in their 3′ untranslated regions (3′UTRs) (Fan and Steitz, 1998; Peng *et al*., 1998). HuR binds to some UC-rich motifs (Yeap *et al*., 2002; Wein *et al*., 2003), and U-rich regions with high affinity for HuR are described (Lopez de Silanes *et al*., 2004; Meisner *et al*., 2004; de Boer *et al*., 2006). HuR stabilizes mRNAs in the cytoplasm, but also protects its target RNAs in the nucleus from entering the exosome, thus avoiding degradation (Moore, 2002).

HuR may have a role in cancer biology. HuR stabilizes the mRNAs of many genes relevant to cancer (Fan and Steitz, 1998; Peng *et al*., 1998; Nabors *et al*., 2001). Upregulation of HuR occurs in several malignant tumors (Nabors *et al*., 2001; Erkinheimo *et al*., 2003; Lopez de Silanes *et al*., 2003). In breast cancer, cytoplasmic HuR is associated with higher tumor grade (Denkert *et al*., 2004; Heinonen *et al*., 2005). In one study, this was associated with a reduced distant disease-free survival (Heinonen *et al*., 2005).

We and others have found that the c-fms proto-oncogene is expressed by the tumor epithelium in several human epithelial cancers (Kacinski *et al*., 1991; Chambers *et al*., 1997; Ide *et al*., 2002); its activation or overexpression confers invasive and metastatic properties in breast cancer (Sapi *et al*., 1996; Lin *et al*., 2001; Toy *et al*., 2005). In human breast cancer, 94% of *in situ* and invasive lesions express c-fms (Kacinski *et al*., 1991). Expression confers an increased risk for local relapse (Maher *et al*., 1998). In a large cohort breast cancer tissue array (Kluger *et al*., 2004), c-fms is strongly associated with lymph node metastasis and poor survival.

We reported that physiological concentrations of glucocorticoids upregulate c-fms expression by up to 50-fold in breast cancer cell lines (Kacinski *et al*., 1991; Chambers *et al*., 1994; Sapi *et al*., 1995). These results were confirmed by using a primary organ culture system of fresh breast-carcinoma specimens (Kacinski *et al*., 2001). Using a mouse model, we show the effect of endogenous circulating glucocorticoids on stimulation of c-fms expression in metastatic implants of human breast cancer (Toy *et al*., 2005). Fifty-two per cent of human breast-cancer tissues have functional glucocorticoid receptors (similar to estrogen receptor (53%))

Correspondence: Dr SK Chambers, Arizona Cancer Center, University of Arizona, PO Box 245024, Tucson, AZ 85724-5024, USA. E-mail: schambers@azcc.arizona.edu
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(Allegra *et al*., 1979), allowing for breast cancer responsiveness to circulating, endogenous glucocorticoids.

Control of c-fms expression is related to both transcriptional and post-transcriptional events (Weber *et al*., 1989; Chambers *et al*., 1993, 1994, 2004; Sapi *et al*., 1995). In monocytes, we have found that dexamethasone (dex) alters c-fms mRNA transcript half-life (Chambers *et al*., 1993); in breast cancer cells, both mechanisms regulate c-fms expression (Chambers *et al*., 1994; Sapi *et al*., 1995). Post-transcriptional events are frequently modulated by protein binding to 3′UTR mRNA; yet, the existence and identity of the c-fms RNA regulatory protein/s remain unknown. The 3′UTR of c-fms does not contain AREs or U-rich regions, described for HuR binding (Lopez de Silanes *et al*., 2004; Meisner *et al*., 2004). There are some relatively UC-rich stretches, although they do not contain the UC-rich motifs described earlier for HuR binding, such as CU_nC or $(CUUU)_{11}(U)_8$ (Yeap *et al*., 2002; Wein *et al*., 2003).

We first systematically studied the role of nuclear and cytoplasmic HuR in the largest cohort to date of 670 breast-cancer patients. High nuclear, but not cytoplasmic, HuR was an independent poor prognostic factor; HuR's expression correlated with c-fms expression. We hypothesized that HuR may bind the 3′UTR of c-fms mRNA and stimulate c-fms expression post-transcriptionally. HuR binding to c-fms mRNA in the nucleus and cytoplasm of breast-cancer cells was shown *in vivo*. HuR bound to a 69-nt fragment of the 3′UTR of c-fms mRNA containing five 'CUU' motifs. We show both by overexpressing and silencing HuR, that regulation of c-fms expression by HuR was primarily at the RNA level. Glucocorticoid stimulation of c-fms RNA and protein expression was largely dependent on HuR. Silencing HuR decreased reporter RNA and activity only in the presence of the wild-type, but not mutant, 69-nt 3′UTR c-fms sequence. Thus, post-transcriptional regulation of c-fms by HuR is dependent on a non-AU-rich element in the 3′UTR c-fms RNA.

Results

HuR is mainly localized to the nucleus of human breast-cancer cells

HuR is a shuttling protein that moves its target mRNAs from the nucleus to the cytoplasm (Fan and Steitz, 1998). By western blotting in three breast-cancer cell lines (Figure 1), HuR was detected predominantly in the nucleus. Cytosolic expression was barely detectable in two of the three cell lines, with MDA-MB-231 cells expressing 0.5-fold less HuR in the cytoplasm than in the nucleus.

HuR immunohistochemical staining of a tissue microarray cohort of 670 breast-cancer patients

This tissue microarray cohort of 670 breast-cancer patients has been used to study the clinical role of Her2/neu and c-fms in breast cancer (Camp *et al*., 2003; Kluger *et al*., 2004). All the 10 control cores of normal breast tissue had low nuclear HuR scores and 8 of the 10 had low cytoplasmic HuR scores. Of 670 breast cancer cores, 593 cores (88.5%) had associated survival information, and 590 cores (88.0%) were interpretable for nuclear and cytoplasmic immunoreactivity. Spots deemed non-interpretable had insufficient tumor cells in the spot, loss of tissue or abundant necrosis. Overall, 74.5% of specimens had strong nuclear immunoreactivity for HuR, with 74.7% having strong cytoplasmic immunoreactivity. In all, 63.6% had strong immunoreactivity for both nuclear and cytoplasmic HuR. Expression of nuclear HuR was strongly associated with cytoplasmic HuR ($P<0.0001$), in line with HuR's known nuclear–cytoplasmic shuttling function. Representative examples of HuR staining are shown in Figure 2.

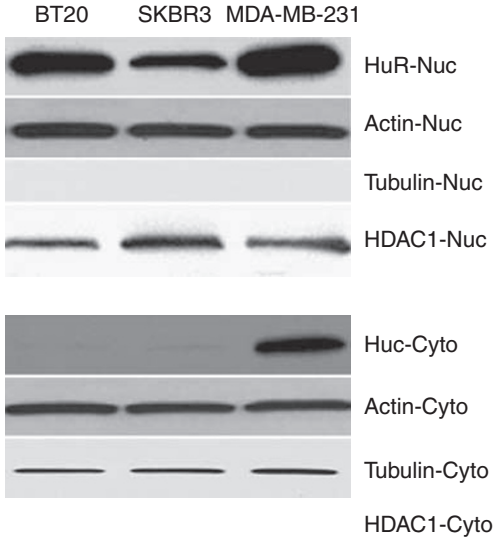

Figure 1 HuR is expressed predominantly in the nucleus of breast-cancer cells. Western blot analysis of the subcellular distribution of HuR in nuclear and cytoplasmic protein extracts, using a mouse anti-human HuR mAb. Pan actin was used as a loading control. The presence of α-tubulin in the cytoplasmic extract only, and the presence of HDAC1 in the nuclear extract only, confirmed the purity of the preparations.

Association of HuR with survival

Kaplan–Meier survival curves showed that high nuclear HuR immunoreactivity was strongly associated with decreased overall survival (log rank $P=0.0025$) (Figure 2 and Table 1). High nuclear HuR was also associated with positive nodal status ($P=0.0371$). Even among the subgroup of lymph node-positive breast-cancer patients, high-nuclear HuR levels retained an association with poor prognosis ($P=0.035$); there was a trend towards this finding ($P=0.08$) in lymph node-negative breast-cancer patients. There was no significant correlation between HuR cytoplasmic staining and survival ($P=0.1073$), even when analysed within nodal subgroups (neither lymph node-positive ($P=0.1154$) nor lymph node-negative cases ($P=0.6426$)).

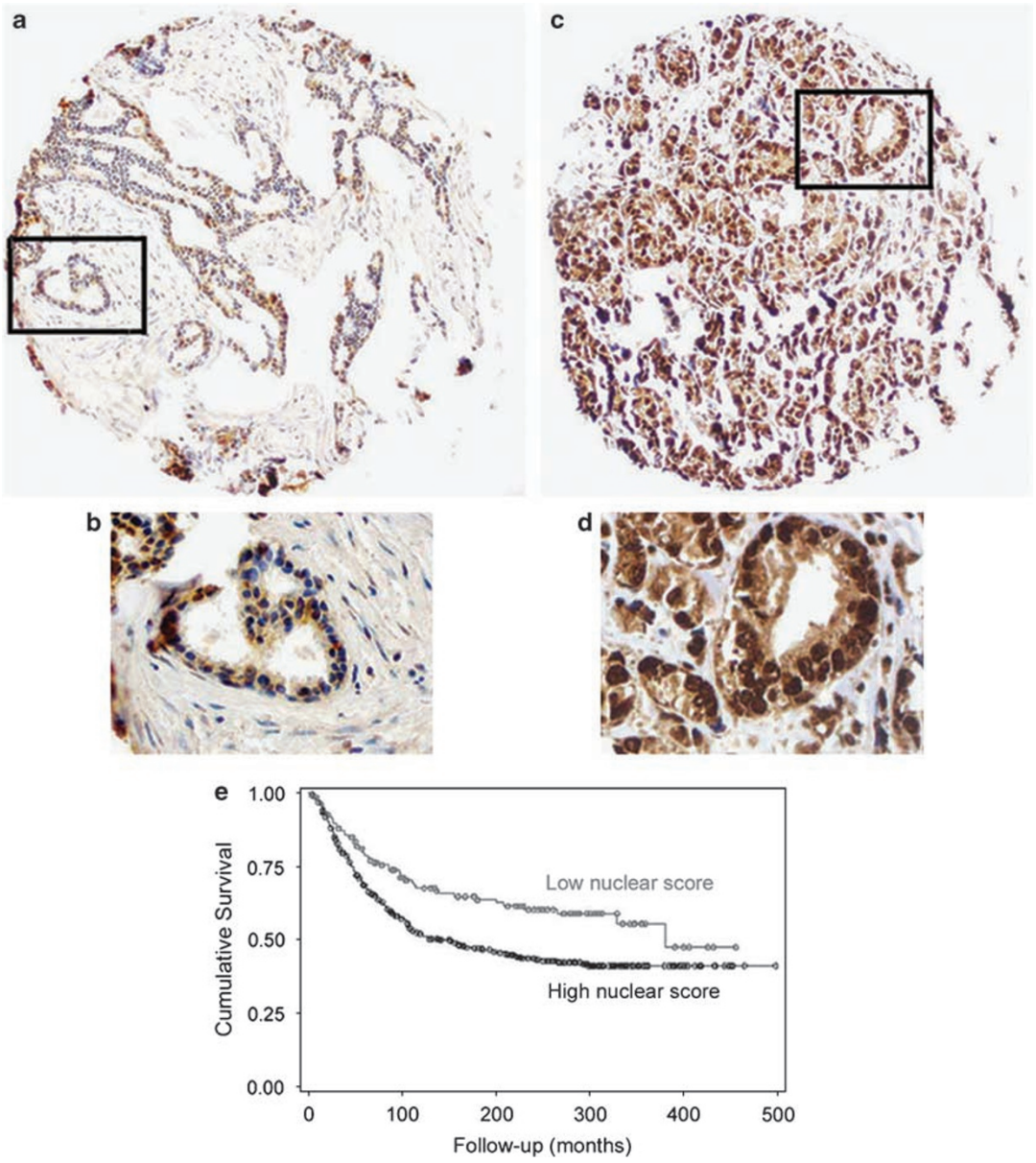

Figure 2 Nuclear HuR is predictive of poor breast cancer survival. (**a**) Intermediate power and (**b**) high power: a tissue core from an adenosis (benign) control sample, with low nuclear and low cytoplasmic staining. (**c**) Intermediate power and (**d**) high power: a tissue core derived from an invasive ductal carcinoma, showing high-nuclear and high-cytoplasmic staining. Original magnifications for panels (**a**) and (**c**): $\times 100$, boxes indicate the areas magnified; their corresponding high magnifications (**b** and **d**): $\times 400$. (**e**) Kaplan–Meier survival curves for HuR nuclear staining of tissue arrays. Strong nuclear staining of HuR was markedly associated with decreased overall survival of breast cancer patients ($P = 0.0025$).

Table 1 HuR expression and survival

HuR expression	*No. of cases*	*No. of events*	*Median survival time in months (95% confidence interval)*	*Mean survival time (months ± s.e.)*	P-*value (log rank)*
Nuclear HuR expression					**0.0025**
Low	137	51	380.2 (265.6–ND)	256.4 ± 14.0	
High	401	210	151.4 (107.4–220.2)	168.0 ± 6.3	
Cytoplasmic HuR expression					0.1073
Low	131	58	329.1 (138.0–ND)	206.2 ± 12.2	
High	387	198	154.7 (109.9–264.4)	170.3 ± 6.4	

Abbreviations: ND, not reached; s.e., standard error.
Bold indicates statistically significant values.

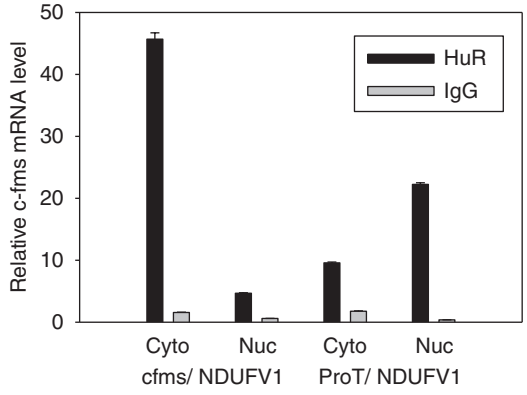

Figure 3 c-fms mRNA is a direct target of HuR protein *in vivo*. After immunoprecipitation (IP) of RNA-protein complexes from BT20 cell cytoplasmic (Cyto) or nuclear (Nuc) lysates, using either anti-human HuR antibody or control IgG, RNA was isolated and used in reverse transcription (RT) reactions and amplified by real-time PCR. NDUFV1 is not a HuR target and served as a negative control, whereas prothymosin (ProTα) mRNA, a known HuR target, was used as a positive control. The graph shows relative mRNA levels in HuR IP compared with control IgG IP conditions, as measured by qRT–PCR analysis. The mean ± s.d. of c-fms or ProTα RNA normalized for NDUFV1 RNA is depicted.

Association of nuclear HuR with clinicopathological parameters

In addition to the association between high nuclear HuR expression and nodal status, HuR was significantly associated with high nuclear grade ($P = 0.0003$) (Table 2). There was no association between increased nuclear HuR and age at diagnosis, large number (>3) of positive nodes, tumor size, negative estrogen or progesterone receptor, or positive Her2/neu expression.

HuR nuclear expression is an independent poor prognostic factor

We performed multivariate survival analysis, using tumor size, nodal status, number of involved lymph nodes, nuclear HuR expression, nuclear grade, and expression of estrogen and progesterone receptor (all found on univariate analysis to be predictive of survival) as parameters. Increased nuclear expression of HuR was an independent prognostic parameter for decreased survival, with a relative risk of 1.448 ($P = 0.0319$, 95% confidence interval, 1.103–1.793). Other independent parameters were tumor size ($P < 0.0001$), nodal status ($P = 0.0115$) and large number (>3) of involved lymph nodes ($P = 0.0219$).

Breast cancers, which strongly express HuR, also strongly express c-fms

The prognostic role of c-fms expression in the cytoplasm in the same patient cohort by tissue microarray was reported earlier (Kluger *et al*., 2004). We found a highly significant association between high nuclear HuR and high cytoplasmic c-fms expression ($P = 0.0007$). A weaker association occurred between high cytoplasmic HuR and high cytoplasmic c-fms expression ($P = 0.0267$).

Table 2 Relationship of nuclear HuR expression with various clinicopathological factors in breast cancer patients

Clinicopathological factor	*Number of cases*	*High HuR nuclear expression (%)*	P-*value*
Age of diagnosis (years)	609		0.5270
⩽57		227/298 (76.2%)	
>57		230/311 (73.9%)	
Nuclear grade	549		**0.0003**
1, 2		282/391 (72.1%)	
3		137/158 (86.7%)	
Number of nodes involved	609		0.1060
⩽3		337/459 (73.4%)	
4–41		120/150 (80.0%)	
Tumor size (cm)	609		0.4878
⩽3		265/358 (74.0%)	
>3		192/251 (76.5%)	
Nodal status	596		**0.0371**
Negative		210/294 (71.4%)	
Positive		238/302 (78.8%)	
Estrogen receptor	583		0.4655
Negative		263/347 (75.8%)	
Positive		185/236 (78.4%)	
Progesterone receptor	666		0.9371
Negative		264/343 (77.0%)	
Positive		271/323 (76.7%)	
HER2	575		0.3182
Negative		359/471 (76.2%)	
Positive		84/104 (80.7%)	

Bold indicates statistically significant values.

c-fms mRNA is a direct target of HuR in breast-cancer cells

As HuR was strongly associated with c-fms in human breast-cancer specimens, and c-fms can be post-transcriptionally regulated, we hypothesized that HuR may regulate c-fms expression by binding to the 3′UTR of c-fms RNA. To test whether HuR directly associates with c-fms mRNA in breast cancer cells, immunoprecipitation (IP) assays using anti-HuR antibody were performed under conditions that preserved ribonucleoprotein integrity. The association of c-fms mRNA with HuR was determined by isolating RNA from the IP material and conducting qRT–PCR analysis. The c-fms PCR product was dramatically enriched in HuR IP samples compared with control IgG IP samples, in both nuclear and cytoplasmic fractions (Figure 3). In BT20 cells, association of 3′UTR c-fms mRNA with HuR was 29.2-fold higher than in control IgG IP reaction in the cytoplasmic fraction and 7.8-fold higher in the nuclear fraction (Figure 3; $P < 0.0001$). NDUFV1 RNA does not interact with HuR and amplification of NDUFV1 PCR products monitored the evenness of sample input (Lopez de Silanes *et al*., 2004). Prothymosin α (ProTα) RNA interacts with HuR (Abdelmohsen *et al*., 2007). Enrichment of ProTα PCR product was also observed in both cytoplasmic and nuclear fractions of BT20 cells ($P < 0.0001$; Figure 3).

We confirmed this significant association of 3′UTR c-fms mRNA with HuR in another breast-cancer cell line, SKBR3 ($P<0.0001$), with larger amounts of c-fms mRNA associated with HuR in the cytoplasm than in the nucleus (not shown). Hence, HuR interacts with 3′UTR c-fms mRNA *in vivo* in both cytoplasmic and nuclear compartments. This interaction with 3′UTR c-fms mRNA is not limited to one breast-cancer cell line.

HuR binds 3′UTR c-fms RNA in vitro

Ultraviolet cross-linking and label-transfer assays were used to determine sequences within the 3′UTR of c-fms RNA interacting with HuR. The 3′UTR of c-fms (3217–3992 nt) (Coussens *et al*., 1986) is neither AU- or U-rich, nor contains the UC-rich motifs described earlier for HuR binding (Yeap *et al*., 2002; Wein *et al*., 2003). However, in the middle of the c-fms 3′-full-length UTR RNA (776 nt; Figure 4a) are five 'CUU' motifs. The riboprobe made from this 776-nt sequence bound recombinant HuR–GST protein (Figure 4b).

HuR binds to a 69-nt element within the 3′UTR c-fms RNA

To identify sequences mediating HuR binding, we tested successively smaller regions of 3′UTR c-fms (Figure 4a): 578-nt c-fms (3018–3992 nt), 218-nt c-fms (3415–3632 nt), 99-nt c-fms (3488–3587 nt) and 69-nt c-fms (3499–3568 nt) RNAs, for HuR protein binding. All retained the capacity for binding HuR (Figures 4b and c).

This suggests that the HuR protein c-fms RNA-binding site is contained in part within the 69-nt 3′UTR c-fms sequences. This 69-nt c-fms sequence has no homology to known human sequences, and has not been described earlier to be a RNA consensus sequence for protein binding.

Mutations in the 69-nt element of 3′UTR c-fms abrogate HuR binding

This 69-nt c-fms sequence (^{3499}CUAGUAGAAC**CUU**-**CUU**UCCUAAUCCC**CUU**AU**CUU**CAUGGAAAUG GACUGA**CUU**UAUGCCUAUGAAGUCC^{3567}) has five 'CUU' motifs (underlined), which are significantly less U-rich than those UC-rich motifs described earlier for HuR binding (Yeap *et al*., 2002; Wein *et al*., 2003). As all five 'CUU' motifs are contained in the 69-nt element, deletion of this 69-nt element would make the RNA in this region significantly less UC-rich. In fact, deletion of this 69-nt c-fms element (3499–3567 nt) (218Δ69 nt) containing all five 'CUU' motifs abrogated binding to HuR protein (Figure 4c).

To test whether the 69-nt c-fms element contains part of the binding site for HuR, mutations in the 69-nt c-fms element were generated and HuR-binding assay was carried out by ultraviolet cross-linking. Three regions that collectively contained all five 'CUU' motifs (3509–3515 nt, 3525–3535 nt and 3546–3554 nt) were mutated. Changing U's to G's in each region abrogated HuR binding (Figure 4c), showing the specificity of interaction between HuR protein and non-AU-rich 3′UTR c-fms RNA. Non-consensus 'CUU' motifs found in the 3′UTR 69-nt c-fms element seem to be critical for HuR binding.

HuR overexpression increases c-fms RNA and protein levels in breast-cancer cells

To demonstrate a functional significance of HuR binding to 3′ UTR c-fms, we overexpressed HuR in BT20 breast-cancer cells. Overexpression significantly increased the c-fms RNA level in both dex-treated (by 47-fold) and untreated (by 55-fold) cells (Figures 5a and c). Figure 5b confirms the overexpression of HuR protein, and shows the increase in c-fms protein by HuR, under dex-stimulated conditions. Under resting (no dex) conditions, bands on western blot were barely detectable (not shown), in line with other reports (Kacinski *et al*., 1991; Chambers *et al*., 1994; Sapi *et al*., 1995). These findings were duplicated in another breast-cancer cell line, SKBR3 (Figures 5d–f). Thus, HuR stimulates c-fms RNA expression in breast-cancer cells. Under dex-stimulated conditions, the increase in c-fms protein by HuR occurs to a lesser degree than that observed at the RNA level.

HuR shRNA downregulates c-fms RNA and protein expression in BT20 breast-cancer cells

We next showed that HuR short hairpin RNA (shRNA) reduced the ability of dex to stimulate c-fms RNA and protein (Figures 6a and b), compared with control vector. We confirm c-fms protein stimulation by dex (control lanes, compare Figures 6b and d). The c-fms RNA levels were reduced by 233-fold, and c-fms protein levels became undetectable, by HuR shRNA under dex-stimulated conditions compared with controls. Under resting conditions, there was a 29-fold reduction in c-fms RNA levels by HuR shRNA (Figure 6c). The c-fms protein was barely detectable under resting conditions (Figure 6d), thus, the effect of HuR shRNA is hard to visualize. Although off-target effects of HuR shRNA could be contributory, c-fms RNA expression in BT20 breast-cancer cells was significantly regulated by HuR, consistent with the significant downregulation of c-fms protein by HuR shRNA under dex-stimulated conditions. Further, these data suggest that glucocorticoid stimulation of c-fms is largely dependent on HuR, as silencing of HuR abrogated dex stimulation of c-fms RNA and protein expression.

HuR promotes reporter activity through post-transcriptional interaction with the 69-nt 3′UTR c-fms element in vivo

HuR binds specifically to a 69-nt element within the c-fms RNA (Figure 4). We reasoned that this binding is important for HuR's regulation of c-fms mRNA and protein (Figures 5 and 6). The 69-nt 3′UTR c-fms element was cloned in the 3′UTR of a luciferase reporter vector (69wt; Figure 7a). After HuR small interfering RNA (siRNA) transfection, luciferase activity decreased by 35% compared with control siRNA-transfected

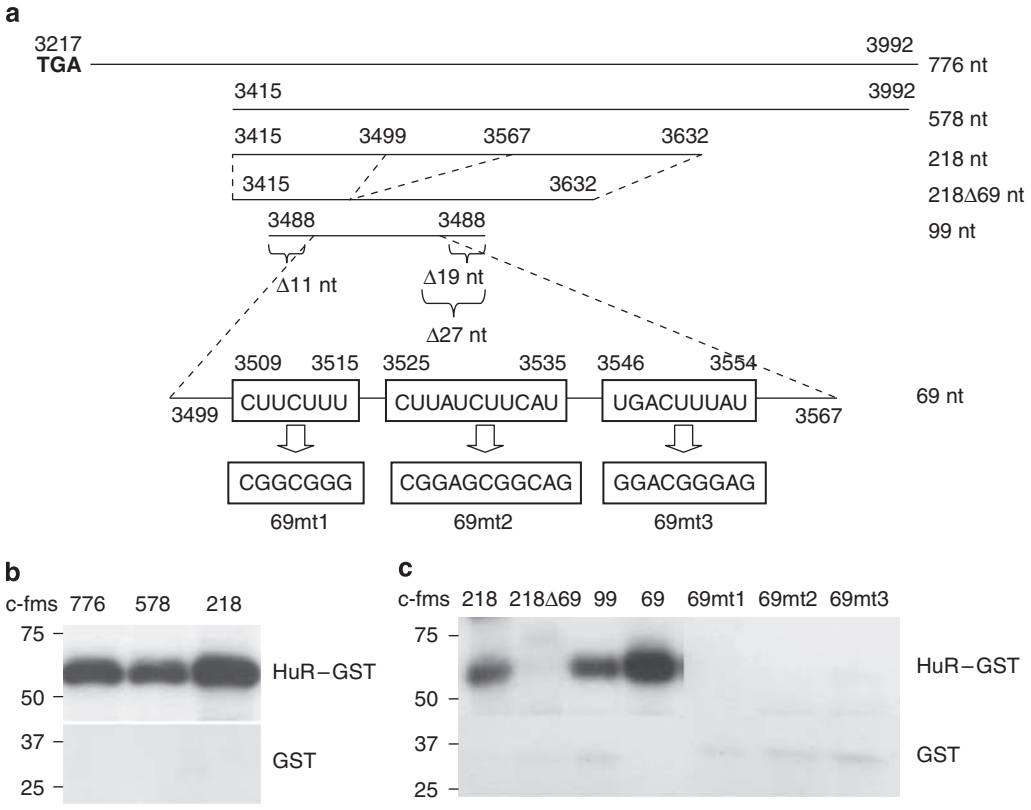

Figure 4 HuR specifically binds 69-nt 3′UTR c-fms containing 'CUU' motifs. (**a**) c-fms 3′UTR mRNA showing 776, 578, 218, 99 and 69 nt regions. Δ indicates deletion in 218 nt. In 69mt1, 2 and 3, U's in three regions within the 69 nt wild-type element were changed to G's, respectively, as depicted. (**b**) Ultraviolet (UV) cross-linking and label transfer of RNA sequences within the 776, 578 and 218-nt 3′UTR of c-fms to HuR–GST, but not to glutathione S-transferase (GST). (**c**) UV cross-linking and label transfer of RNA sequences within the 3′UTR of c-fms in the presence or absence of the 69-nt c-fms element, or mutations within this 69-nt element, to HuR. The binding complex of 218-nt c-fms (3415–3632 nt), 99-nt c-fms (3488–3586 nt), and 69-nt c-fms (3499–3567 nt) to recombinant HuR–GST was detected at 55–60 kDa. Mutations (U's to G's) in each of the three regions within the 69-nt element abrogated HuR binding. No binding of 218 nt Δ69 nt (3415–3632 nt with 69 nt (3499–3567 nt) deleted) to recombinant HuR–GST was detected. GST, used as a negative control, did not bind 218-nt (3415–3632 nt, 99-nt (3488–3586 nt) or 69-nt c-fms (3499–3567 nt).

cells ($P = 0.039$) (Figure 7b). As expected, since regulation by the interaction of HuR with the 69-nt wild-type c-fms element is thought to be post-transcriptional, luciferase RNA levels corresponded with luciferase activity, that is 27% reduction of luciferase RNA in HuR siRNA compared with control siRNA transfected cells ($P < 0.001$). In contrast, in a luciferase construct containing the 69 nt c-fms mutant sequence (69 mt; in which all U's were changed to G's as depicted, Figure 7a), no difference in luciferase activity occurred between HuR siRNA- and control siRNA- transfected cells, similar to the luciferase vector control (Figure 7b). Under control siRNA conditions, even with one copy of the 69-nt c-fms wild-type (69 wt) sequence, a 3.1-fold increase in luciferase activity was seen, compared with the 69-nt mutant (69 mt) sequence ($P = 0.003$).

These data suggest that HuR specifically interacts with 69-nt 3′UTR c-fms RNA, resulting in upregulation of gene expression at a post-transcriptional level. The 69-nt wild-type c-fms element, but not the mutant, promotes gene expression. HuR may be only one of the RNA-binding proteins that mediate this effect, as HuR siRNA did not completely abrogate the increase in luciferase activity by the 69-nt wild-type sequence, although the difference under HuR siRNA conditions between 69 wild-type and 69 mt sequences was not significant ($P = 0.135$).

Discussion

We hypothesized that HuR would play a significant biological role in human breast cancer. We systematically investigated the role of both nuclear and cytoplasmic HuR, as HuR is a nucleocytoplasmic shuttling protein, in what we believe to be the largest breast-cancer cohort tissue array to date to be used for this purpose. In a recent immunohistochemical study of HuR in 208 primary breast carcinomas, neither nuclear nor cytoplasmic HuR correlated with nodal status or patient survival. High-nuclear HuR was found in 61%, with high cytoplasmic HuR in 30% of cases (Denkert *et al*., 2004). Another study of HuR in 133 breast-cancer specimens showed that high cytoplasmic HuR correlated with poor distant disease-free survival but not

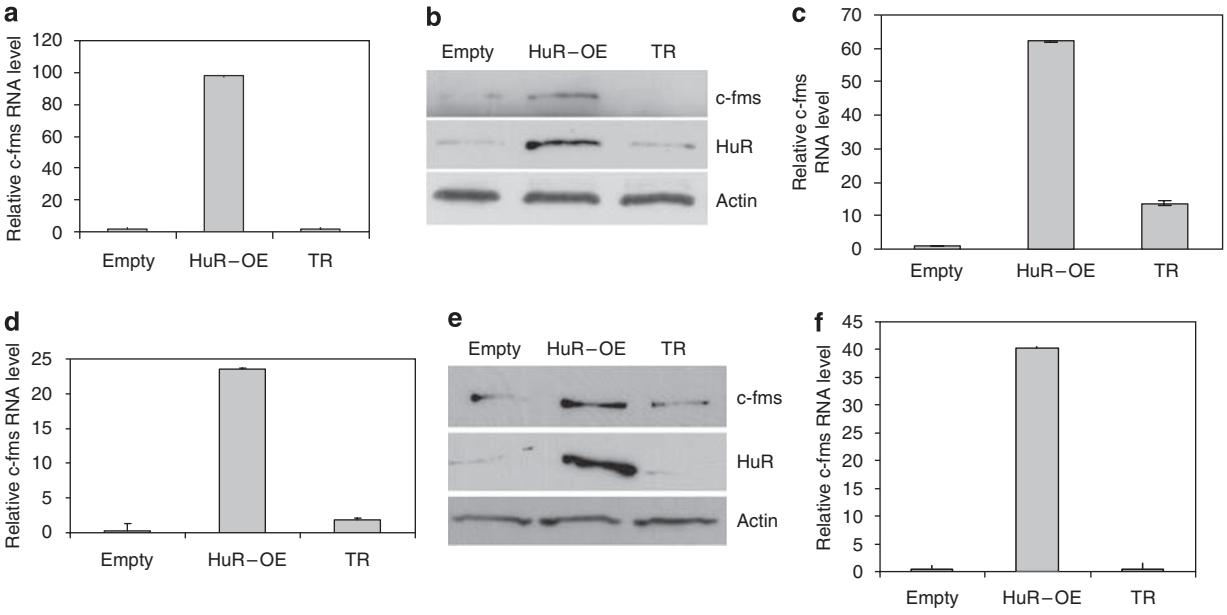

Figure 5 HuR overexpression increases c-fms RNA and protein expression in BT20 (**a**, **b**, **c**) and SKBR3 (**d**, **e**, **f**) breast-cancer cells. qRT–PCR analysis shows a significant effect of HuR overexpression on c-fms RNA levels (mean ± s.d.) in BT20 (**a**) and SKBR3 (**d**) cells treated with 100 nM dex. (**b** and **e**) Western blot analysis confirms the increase in HuR protein and shows the increase in c-fms protein by HuR overexpression in dex-treated cells. (**c** and **f**) qRT–PCR analysis shows the effect of HuR overexpression on c-fms RNA in cells in the absence of dex. Negative controls include the empty vector and transfection reagent (TR) conditions.

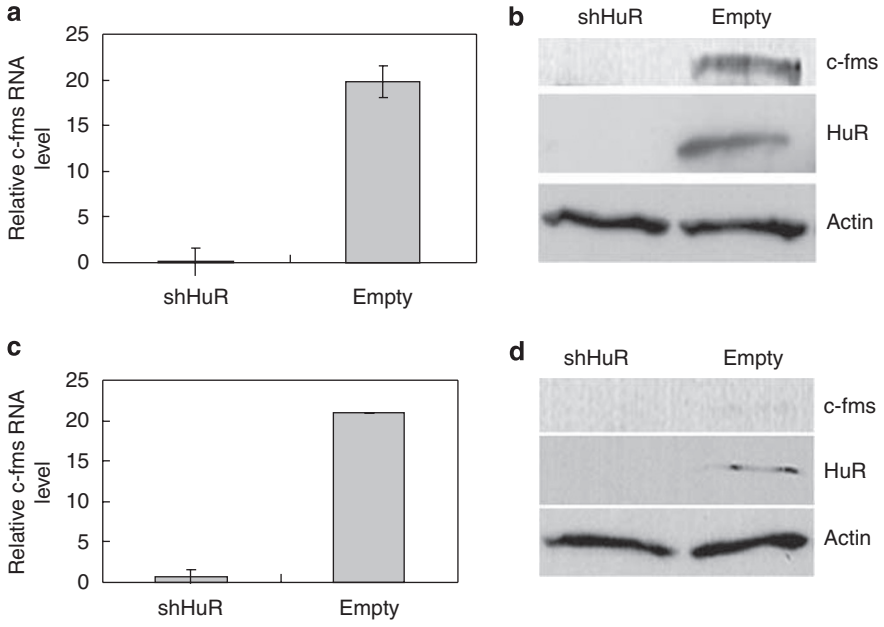

Figure 6 Silencing HuR downregulates c-fms RNA and protein expression in BT20 breast-cancer cells. qRT–PCR analysis of (**a** and **c**) c-fms RNA expression in HuR short hairpin RNA (shRNA) or empty vector (empty)-treated BT20 cells, in the presence (**a**) or absence (**c**) of 100 nM dex for 96 h. (**b** and **d**) Western blot analysis of cytoplasmic lysates from HuR shRNA or empty vector (empty)-treated BT20 cells in the presence (**b**) or absence (**d**) of 100 nM dex for 96 h. The membrane was probed with antibodies to c-fms, HuR and Pan-actin. (**a, b**) HuR–shRNA treatment was able to eradicate dex-stimulated c-fms RNA and protein. (**c**) HuR–shRNA also significantly reduced c-fms RNA under resting conditions. (**d**) c-fms protein was barely detectable under resting (no dex) conditions, as reported earlier. The film was overexposed to show HuR protein in the empty vector-transfected cells.

nodal status (Heinonen *et al*., 2005). In that study, the role of nuclear HuR could not be evaluated. In contrast, in our cohort of 670 breast-cancer specimens, nuclear, but not cytoplasmic, expression of HuR was an independent poor prognostic factor (Figure 2). Differences in results between our study and others could

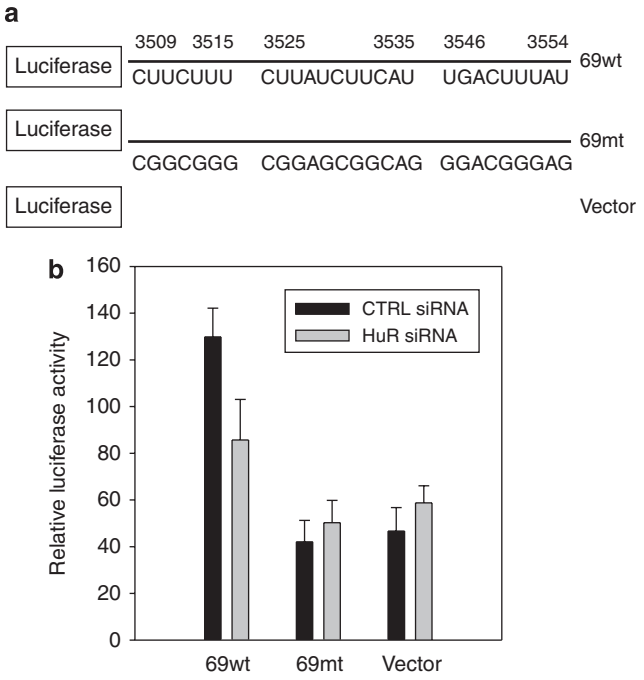

Figure 7 HuR post-transcriptionally regulates reporter gene expression by interacting with the 69-nt 3′UTR c-fms element. At 48 h after HuR–small interfering (si)RNA transfection, BT20 cells were transfected with the indicated luciferase constructs. Relative luciferase activity is the ratio between *firefly* luciferase and *renilla*-control luciferase, adjusted to 100%. Control siRNA-transfected cells were also transfected with the indicated luciferase constructs for comparison. (**a**) Luciferase constructs containing in the 3′UTR: wt = 69-nt wild-type 3′UTR c-fms sequence, mt = 69-nt mutated sequence, and empty vector. (**b**) Luciferase activity with the 69 wild-type construct was decreased by 35% in HuR siRNA compared with control siRNA-transfected cells ($P = 0.039$). No significant changes in luciferase activities with the 69mt or empty vector were observed after HuR–siRNA treatment. Under control siRNA conditions, the 69 wt sequence increased luciferase activity by 3.1-fold ($P = 0.003$) compared with the 69 mt sequence.

reflect differences in methods, antibody used, scoring applied or statistical power. We found that high nuclear HuR was significantly associated with both high nuclear grade and lymph node metastasis, findings that are in line with nuclear HuR's association with poor patient outcome.

Earlier studies localized HuR predominantly (90%) to the nucleus of most unstimulated cells (Lal *et al*., 2004). In breast-cancer cells, we confirm this observation (Figure 1). In breast-cancer specimens, nuclear HuR staining was detected in 74% of cases with the majority (85%) having concurrent strong cytoplasmic staining, underlying the shuttling function of the HuR protein *in vivo*. The influence of HuR on target mRNA stabilization has been linked to HuR's cytoplasmic presence. However, blocking HuR shuttling to the cytoplasm does not interfere with its ability to stabilize c-fos mRNA (Chen *et al*., 2002). As mRNA stabilization can occur in both nuclear (Moore, 2002) and cytoplasmic compartments, it makes sense that nuclear HuR could have such a strong impact on patient outcome. In fact, in breast-cancer cells, we show that c-fms mRNA is a direct target of HuR in both cytoplasmic and nuclear compartments (Figure 3). Further, we find both cytoplasmic and nuclear HuR associated with cytoplasmic c-fms protein in breast-cancer specimens. It is also possible that HuR, which can shuttle out of the nucleus on stimulation (Gallouzi and Steitz, 2001), may export c-fms mRNA to the cytoplasm where HuR may cooperate with other cytoplasmic proteins in stabilization of target mRNAs (Apponi *et al*., 2007), after which HuR is recycled to the nucleus, where it may reside the majority of the time.

In our breast-cancer tissue microarray cohorts, which are continuously exposed to glucocorticoids *in vivo*, HuR was highly co-expressed with c-fms ($P = 0.0007$). The c-fms expression was shown earlier to be a strong predictor of poor breast cancer survival through the association with nodal metastasis (Kluger *et al*., 2004). Colony-stimulating factor-1, its growth factor ligand, imparts metastatic capabilities to breast-cancer cells (Lin *et al*., 2001). This close association between HuR and c-fms led us to ask whether HuR may bind the 3′UTR of c-fms mRNA and stimulate c-fms expression post-transcriptionally.

HuR exhibits specific affinities for the 3′UTR RNA containing AREs of several proto-oncogenes, cytokines and transcription factors, thereby regulating expression of these mRNAs on a post-transcriptional level (Fan and Steitz, 1998; Lopez de Silanes *et al*., 2004). Overexpression of HuR dramatically increases ARE-binding activity in cells (Peng *et al*., 1998). Recently, evidence has emerged that HuR and other members of the Hu family also have affinities for U-rich or UC-rich RNA regions (Yeap *et al*., 2002; Wein *et al*., 2003; Lopez de Silanes *et al*., 2004; Meisner *et al*., 2004; de Boer *et al*., 2006). First, we confirm HuR binding to c-fms mRNA in the nucleus and cytoplasm of breast-cancer cells *in vivo* (Figure 3). Next we show, for the first time, HuR binding specifically to a 69-nt element in the 3′UTR of c-fms lacking any of the AU-, U- or UC-rich motifs described earlier for HuR binding (Figure 4) (Fan and Steitz, 1998; Peng *et al*., 1998; Yeap *et al*., 2002; Wein *et al*., 2003; Lopez de Silanes *et al*., 2004; Meisner *et al*., 2004; de Boer *et al*., 2006). This 69-nt element has five 'CUU' motifs, with mutations in these 'CUU' motifs disrupting HuR binding. This suggests that 'CUU' motifs are critical for HuR binding to 3′UTR c-fms RNA. This 69-nt c-fms sequence has no homology to known human sequences, and has not been described earlier to be a RNA consensus sequence for protein binding. Reporter gene expression is upregulated in the presence of this 69 nt wild-type, but not mutant, element (Figure 7).

We have shown earlier that c-fms mRNA can be stabilized by post-transcriptional events (Chambers *et al*., 1993). First, we show by HuR overexpression (Figure 5), and by HuR RNA interference (Figure 6), the significant regulation of c-fms RNA by HuR. The effect on c-fms RNA levels by HuR is very large, with a smaller to equivalent effect of HuR on c-fms protein

levels. Thus, in this case, it is extremely unlikely that HuR is functioning at the level of enhancing translation of the c-fms protein (Sureban *et al*., 2007). Collectively, the results of these studies suggest that the primary effect of HuR on c-fms regulation is at the RNA level.

Next, our results show that silencing of HuR abrogated dex stimulation of c-fms RNA and protein expression (Figure 6). When stimulated with dex, c-fms RNA was >200-fold under control conditions compared with HuR shRNA (Figure 6a), accounting for more than the known 50-fold stimulation of c-fms RNA and protein by dex in breast-cancer cells (Kacinski *et al*., 1991; Chambers *et al*., 1994; Sapi *et al*., 1995). HuR shRNA resulted in undetectable c-fms protein levels (Figure 6b). These results indicate that dex-stimulated c-fms RNA and protein expression in breast-cancer cells may be largely dependent on the presence of HuR. As human breast-cancer cells are exposed continuously to glucocorticoids *in vivo*, this lends support for HuR's supportive role for c-fms in breast cancer progression.

Lastly, we show that HuR, by binding the 69-nt 3′UTR c-fms wild-type, but not mutant, sequence, promotes reporter gene expression post-transcriptionally (Figure 7). Reporter regulation by HuR (Figure 7) is not of the same magnitude as that of endogenous c-fms (Figures 5 and 6). There are several potential explanations, including limitations of this reporter model, in which one copy of the 69-nt c-fms element was expressed in the absence of neighboring sequences to study changes in reporter expression. Sometimes, the context of neighboring sequences is important to promote proper RNA folding for optimal protein recognition *in vivo*. Another possibility is that although HuR binding to the 69-nt element regulates c-fms expression, there may be other mechanisms that exist for HuR regulation of c-fms in breast-cancer cells.

Our results show for the first time that: (1) nuclear HuR is an independent prognostic factor for poor survival in breast cancer, with both nuclear and cytoplasmic HuR being co-expressed with c-fms in these breast cancers, (2) binding between HuR protein and c-fms mRNA is shown *in vivo* in breast-cancer cells in both nuclear and cytoplasmic compartments, (3) HuR binds specifically to a 69-nt element with 'CUU' motifs in 3′UTR c-fms mRNA, which encodes an oncoprotein shown to confer invasive and metastatic properties in breast cancer, (4) HuR significantly increases c-fms RNA levels in the presence or absence of glucocorticoids, (5) glucocorticoid stimulation of c-fms RNA and protein is largely dependent on HuR, (6) the 69-nt element of c-fms RNA promotes gene expression and (7) HuR binding to this non-AU-rich 69-nt element of 3′UTR c-fms RNA promotes gene expression post-transcriptionally.

Collectively, our findings suggest that HuR plays a supportive role for c-fms in breast cancer progression by binding a 69-nt non-AU-rich element in its 3′UTR, thus regulating its expression.

Materials and methods

Human breast tissue microarray construction

Tissue microarrays were constructed by the Yale University Cancer Center Tissue Microarray Shared Resource as described earlier, with approval from the Yale University Institutional Review Board, and validated before use (Camp *et al*., 2000, 2003; Kluger *et al*., 2004).

Immunohistochemical analysis of HuR

Immunohistochemical analysis was performed using the streptavidin-biotin-peroxidase technique with a HuR monoclonal antibody (IgG_2, clone 19F12, Molecular Probes, Carlsbad, CA, USA). HuR antigen was unmasked using the microwave-mediated antigen retrieval method described earlier (Camp *et al*., 2003). Sections from conventional invasive breast carcinoma served as positive controls for HuR staining. Negative controls were prepared by replacing primary antibodies with class-matched mouse IgG immunoglobulins on parallel sections. To confirm the specificity of the HuR staining, 10 additional breast specimens were restained with or without the antigenic peptide for 19F12 (10 μg/ml, Molecular Probes) for 2 h at room temperature before the staining procedures. Immunostaining was performed twice with identical results.

Evaluation of immunohistochemical staining

Nuclear and cytoplasmic staining was determined separately for each stained tissue core. Staining intensity was graded as follows: 0 = no staining; 1 = weak staining; 2 = moderate staining; and 3 = intense staining. HuR nuclear staining was less uniform than cytoplasmic staining in array spots; hence, scores were generated by multiplying the intensity by the percentage of positive cells in a defined tissue core, yielding scores ranging from 0 to 300. Nuclear scores <150 were defined as 'low' and scores ⩾150 as 'high.' HuR cytoplasmic staining within the array spots was homogeneous as described for c-fms in this breast-cancer tissue microarray (Kluger *et al*., 2004), thus, no area variable was included in the cytoplasmic scoring. Cytoplasmic scores of 0 or 1 were defined as 'low' and scores of 2 or 3 as 'high.' Specimens that were non-interpretable or did not contain carcinoma were not included in the analysis. Scoring was performed by two pathologists (WZ and ECU) in a double-blind fashion. In case of discrepancy between the two pathologists' scores, a consensus score was generated after the slides were reviewed again. The c-fms and Her2/neu staining scores for each specimen were obtained from earlier studies on the same cohort (Camp *et al*., 2003; Kluger *et al*., 2004).

Statistical analysis

All analyses pertaining to the tissue microarray were performed using SAS Proprietary Software version 9.0 (SAS Institute Inc., Cary, NC, USA). Sigmastat (SPSS Inc., Chicago, IL, USA) was used for comparison of differences between groups by analysis of variance or the Student's *t*-test.

In vivo *formaldehyde fixation of cells and cell fractionation*

1×10^8 BT20 or SKBR3 cells were cultured in MEM medium with 10% fetal bovine serum to 80–90% confluence. After trypsin treatment, cells were washed twice with phosphate buffer saline, incubated with formaldehyde (Sigma, St Louis, MO, USA) and diluted by phosphate-buffered saline at a final concentration of 1% for 15 min at room temperature with slow agitation. Glycine was added to a final concentration of 0.25 M to quench the fixation. Cells were washed three times with

ice-cold phosphate buffer saline and collected by centrifugation. Cytoplasmic and nuclear proteins were extracted by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA).

Immunoprecipitation of ribonucleoprotein complexes and reverse transcription and quantitative real-time PCR

The assay was performed as described (Peritz *et al*., 2006). NDUFV1 is not a HuR target (Lopez de Silanes *et al*., 2004) and served as a negative control, whereas ProTα binds to HuR (Abdelmohsen *et al*., 2007) and was chosen as a positive control.

Ultraviolet cross-linking and label transfer of HuR with 3′UTR c-fms RNA

Ultraviolet cross-linking of HuR was performed as described elsewhere (Stolow and Berget, 1990; Gott *et al*., 1991; Urlaub *et al*., 2000), with modifications. RNAs of 3′UTR c-fms labeled with ^{32}P-UTP to the same specific activity were incubated with 0.5 μg HuR–GST or 0.5 μg GST. The 15 μl reaction mixture contained 5 mM HEPES (pH 7.6), 1.25 mM $MgCl_2$, 3.8% glycerol, 0.02 mM DTT, 1 mM EDTA, 25 mM KCl, 50 ng yeast tRNA, 50 ng heparin, 1 mM ATP and ^{32}P-labeled RNA probe (50 000 c.p.m.). After incubation at 30 °C for 15 min, the reaction mixture in a 96-well polystyrene plate on ice was illuminated at 254 nm, 125 mJ for 120 s and 30 mJ for 60 s using a GS Gene Linker UV Chamber (Bio-Rad, Hercules, CA, USA).

shRNA treatment

Plasmids encoding a control shRNA or shRNA (CGTTTGG TGCCGTCACCAATGTGAAAGTG) directed against HuR were purchased from Origene (Rockville, MD, USA). shRNA with partial green fluorescent protein sequence (29 nt) served as a negative control. Cells expressing control shRNA or shRNA targeting HuR were examined for c-fms mRNA and protein abundance in the cytoplasmic lysates of BT20 cells. After 96 h of serum starvation in the absence or presence of 100 nM dex, cells were collected; total RNA was isolated and analysed by qRT–PCR. The data (mean ± s.d.) were averaged from several experiments and standardized to the NDUFV1 mRNA internal control. For protein analysis, cytoplasmic lysates were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockford, IL, USA).

Supplementary information for materials and methods is available on the Oncosene website (http://www.nature.com/onc).

Abbreviations

ARE, AU-rich elements; 3′UTR, 3′untranslated region; dex, dexamethasone; IP, immunoprecipitation; qRT–PCR, reverse transcription and quantitative real-time polymerase chain reaction (real-time PCR).

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