Abstract. Background: The role of human antigen R (HuR) and its prognostic value in breast cancer is currently only partially understood. This study aimed to determine the levels of expression of HuR in breast cancer and assess its prognostic value. Materials and Methods: HuR expression levels were assessed in a cohort of human breast cancer specimens and cell lines using both quantitative and qualitative analysis along with immunohistochemical techniques and the results were compared to the patient details. Results: Immunohistochemical staining revealed a weak HuR staining pattern in breast tumour sections compared to the intense epithelial staining of the normal breast tissues. The levels of HuR transcripts were lower in the more advanced TNM4 and TNM3, poor outcome NPI-2 (Nottingham Prognostic Index) and NPI-3 and node-positive tumours compared with the early stage TNM2 and TNM1, NPI-1, and node-negative tumours, although these values did not reach statistical significance. The patients with metastasis, those who died of breast cancer and those with bone metastasis had significantly decreased levels of HuR transcripts, (p=0.031, p=0.018 or p=0.038 respectively) compared to the disease-free patients. The patients with poor prognosis, including those with metastasis and those who died of breast cancer, had a significantly lower level of HuR transcripts compared to the disease-free patients (p=0.021). High levels of HuR correlated with longer overall survival, although the values did not reach statistical significance (p=0.06). Conclusion: While no significant association of the levels of HuR expression with pathological status of breast cancer is reported, HuR may represent a prognostic factor in human breast cancer, as patients expressing high levels of HuR have a favourable prognosis, and the prognostic role of HuR for breast cancer metastasis, particularly bone metastasis is supported.

Breast cancer affects 1 in 10 women in their lifetime in the United States and the United Kingdom (1, 2). The presence and development of breast cancer is known to be associated with cell division, resistance to apoptosis, maintenance of angiogenesis, tissue invasion, evasion of antitumour immune responses and factors which affect the invasive nature of breast cancer cells. The factors that determine the invasive nature of cancer cells include proto-oncogenes, pro-angiogenes, cell cycle regulators, immunosuppressive cytokines and growth-promoting factors. An important mechanism of post-transcriptional gene regulation of these factors and cytokines is through the control of cytoplasmic mRNA stability mediated by the AU- and U-rich elements (AREs) in its 3’ untranslated region (3’ UTR) (3). Numerous ARE-containing mRNAs encoding proto-oncogenes, cell cycle regulators, cytokines and growth factors have been reported either to be associated with the human (Hu) antigen R (HuR) or, further, to be post-transcriptionally regulated by HuR (4).

HuR, a ubiquitously expressed member of the Hu family of RNA-binding proteins, was first cloned and identified in 1996 (5), and the human HuR gene was localized to human chromosome 19p13.2 (6). The HuR protein family comprises four vertebrate members, the primarily neuronal proteins HuB (Hel-N1), HuC (PLE21) and HuD, and the ~34 kDa protein HuR (6). As members of the Hu protein family share homology with the Drosophila embryonic lethal abnormal vision (ELAV) proteins, they are sometimes regarded as the Hu/elav family (7). In humans, HuR is widely expressed in all proliferating cells (3). The HuR protein contains three highly conserved RNA recognition motifs (RRMs) and a variable basic hinge region between its RRM2 and RRM3 (4, 8). The less conserved hinge region is believed to contain sequences that allow HuR to shuttle between the nucleus and cytoplasm (4). HuR has a variety of biological functions, all of which are based on its ability to bind to and stabilise AREs.
of specific target mRNAs and prevent their degradation, thus, influencing their expression (5, 9).

A variety of target mRNAs, bound and stabilised by HuR, are linked to cancer. Many of such target mRNAs encode cell cycle regulators and proto-oncogenes such as cyclin A, cyclin B1, cyclin D1, c-myc and c-fos (10-12), growth factors, such as epidermal growth factor (EGF) (13) and granulocyte macrophage-colony stimulating factor (GM-CSF) (14), as well as other factors and cytokines that influence tumour progression such as hypoxia inducible factor-1α (HIF-1α) (13), vascular endothelial growth factor (VEGF) (15), urokinase-type plasminogen activator (uPA) (16), matrix metalloproteinase-9 (MMP-9) and metastasis-associated protein 1 (MTA1) (17). Through its association with such target mRNAs, HuR has been found to play a pivotal role in carcinogenesis and subsequent progression via diverse cancer-cell phenotypes; enhanced cell division, resistance of apoptosis, maintenance of angiogenesis, invasion of tissues and metastasis, and evasion of antitumour immune responses (18). In a previous study consisting of approximately 300 paired cancer and normal specimens, it was shown that expression of HuR was increased in a wide variety of human carcinoma tissues compared to the normal tissue counterparts, particularly in colorectal cancer (19).

HuR expression was examined in a cohort of human breast cancer patient specimens in association with patient histological and clinical variables, and the prognostic value of HuR was also investigated.

Materials and Methods

Cell lines and culture conditions. The MCF7, MDA-MB-231 and ZR-751 breast cancer cell lines used in this study were obtained from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK). The cells were routinely cultured in DMEM/Ham’s F12 with L-Glutamine medium (PAA Laboratories, Somerset, UK), supplemented with streptomycin, penicillin and 10% foetal calf serum (PAA Laboratories) and incubated at 37.0°C, 5% CO₂ and 95% humidity.

Human breast specimens. A total of 143 breast samples were obtained from breast cancer patients (34 were background normal breast tissue and 109 were breast cancer tissue). These tissues were collected immediately after mastectomy and snap frozen in liquid nitrogen with the approval of the local ethical committee. The background normal mammary tissues were removed from the same patients, but away from the tumours. The pathologist verified the normal background and cancer specimens, and it was confirmed that the background samples were free from any tumour deposit. The median follow-up for the cohort was 6 years (May 2003). For patient clinical data, see Table I.

Total cellular RNA preparation. The total cellular RNA was isolated from the homogenized breast samples and human cell lines using TRI reagent (Sigma, Dorset, UK) in accordance with the supplied protocol. Following extraction, the RNA concentration was quantified through spectrophotometric measurement (WPA UV 1101, Biotech Photometer, Cambridge, UK).

Reverse transcription polymerase chain reaction. cDNA was generated from 250 ng of each RNA sample and reverse transcribed using an enhanced avian reverse transcriptase-PCR-100 kit with anchored oligo (dT) primers (Sigma). The quality of cDNA was verified using primers to detect the GAPDH housekeeping gene (full primer sequences are outlined in Table II). Conventional PCR was performed in a T-Cy Thermocycler (Creacon Technologies Ltd., CD Emmen, Netherlands) using REDTaq ReadyMix™ PCR Reaction Mix (Sigma). A negative control, where cDNA was replaced with water was also included. PCR conditions were as follows: denaturing at 94°C for 40 sec, annealing at 55-58°C for 40 sec and extension at 72°C for 80 sec. PCR was conducted over 38 cycles with an initial 5 min denaturing step (94°C) and a final 10 min extension step (72°C). Following PCR, the products were loaded on a 0.8% agarose gel, separated electrophoretically, stained in ethidium bromide and visualised under ultraviolet light.

| Sample no. | Tissue sample | Normal | 34 | Tumour | 109 |
| Histological grade | | 1 | 20 | 2 | 39 | 3 | 50 |
| TNM staging | 1 | 61 | 2 | 36 | 3 | 7 | 4 | 5 |
| Node status | Node negative | 59 | Node positive | 50 |
| Histological type | Ductal | 92 | Lobular | 13 | Others | 4 |
| ERs status | ER(–) | 75 | ER(+) | 34 | ERβ(–) | 84 | ERβ(+) | 25 |
| Nottingham Prognostic Index (NPI) | 1 (<3.4) | 59 | 2 (3.4-5.4) | 35 | 3 (>5.4) | 15 |
| Clinical outcome | Disease free | 81 | Poor outcome | 5 | With metastasis | 4 | Died of breast cancer | 16 | Died of unrelated disease | 3 |

ER: oestrogen receptor.
Table II. Primer sequences.

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<th>Sense primers (5′-3′)</th>
<th>Antisense primers (5′-3′)</th>
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<tr>
<td>HuR(PCR) 5′-GAGCTCAGAGTTGATCAAG</td>
<td>5′-TTTAGATGAAAAATGCACCA</td>
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<tr>
<td>HuR(Q-PCR) 5′-AGACATGTTCTCTCGGTGTTG</td>
<td>5′-ACTGAACCTGACCGTCAAACTTGTAATTTGCTTCTTG</td>
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<tr>
<td>GAPDH(PCR) 5′-GGCTCGTCTTTAATCTCCTGTA</td>
<td>5′-GACTGTTGTCATGAGTCCT</td>
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Quantitative-polymerase chain reaction (Q-PCR). The iCycler IQ system (Bio-Rad, Hertfordshire, UK) was used to detect and quantify the number of HuR transcripts in the breast specimens, which was then analysed in association with the patient data. The results are given as the number of copies/50 μL based on an internal standard and the results were further normalised using the expression of GAPDH in these samples. The Q-PCR technique used the Amplifluor system (Intergen Inc., Purchase, NY, USA), Q-PCR Master Mix (ABgene, Surrey, UK) and an universal probe (Uniprimer™, Intergen) to record the fluorescence. Conditions for Q-PCR were: an initial 15 min 95˚C period followed by 80 cycles of 95˚C for 15 sec, 55˚C for 60 sec and 72˚C for 20 sec. Full details of the primers used are given in Table II.

Immunohistochemical staining of breast specimens. The frozen sections of breast tumours and background tissues were cut at a thickness of 6 μm using a cryostat. The sections were mounted on super frost plus microscope slides, air dried, and then fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in BSS buffer for 5 to 10 min to rehydrate. Following blocking in a Super Sensitive™ Wash Buffer (BioGenex, San Ramon, CA, USA) containing horse serum, the primary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was added at a concentration of 1:100 and incubated for 60 min. After extensive washing, a Vectastain universal quick kit biotinylated pan-specific secondary antibody (Vector Laboratories, Peterborough, UK) was added in accordance with the provided protocol and incubated for 30 min. Following extensive washings, the avidin/biotin complex (Vector Laboratories) was applied. This was followed by additional washes, the addition of Diaminobenzidine chromogen (Vector Laboratories) to the sections and subsequent incubation, in the dark, for 5 min. Sections were then counterstained in Gill’s haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a coverslip. Staining was independently assessed by the authors.

Statistical analysis. The experimental procedures were repeated independently at least three times. The data was analysed using the Minitab 14 software and SPSS 16 statistical software packages. Statistical comparisons were drawn using the two-sample, two-tailed t-test, Kaplan–Meier survival or Cox proportion analysis. A p-value of <0.05 was taken as significant.

Results

The expression of HuR in human breast cancer. As shown by conventional PCR, HuR was highly expressed in all the breast cancer cell lines (MCF7, MDA-MB-231 and ZR-751) tested (Figure 1A). A decreased level of HuR expression was revealed in the breast tumours (233±92 copies/50 ng RNA) compared to the normal background tissues (395±365 copies/50 ng RNA), although the value did not reach statistical significance (p=0.67; see Figure 1B).

Immunohistochemical staining of HuR in human breast specimens. To further confirm the expression of HuR in human breast tissue, immunohistochemical staining of HuR was undertaken. The HuR protein stained well in the normal breast tissue sections, and its distribution was mainly confined to the intensely stained epithelial cells and absent in the stromal fibroblasts (Figure 2, left). Immunohistochemical staining for HuR was seen, largely, to be negative/weakly positive in the breast tumour cells when compared against the intense epithelial staining of the normal breast tissues (Figure 2, right).

HuR and histological grade, TNM staging, lymph node involvement and histological type. According to the histological grade (grade 1, n=20; grade 2, n=39; grade 3, n=50) of the tumour cells, no obvious difference of HuR expression was seen in the poorly-differentiated grade 2 (309±178 copies/50 ng RNA) and grade 3 (220±135 copies/50 ng RNA), p=0.54 and p=0.78 compared with the well-differentiated grade 1 (162±159 copies/50 ng RNA) (Figure 3A).

The association of the levels of HuR transcripts with TNM staging was also analysed (Figure 3B). The more advanced TNM4 (0.3±0.2 copies/50 ng RNA) and TNM3 (4±3.6 copies/50 ng RNA) tumours had reduced levels of HuR compared with TNM1 (210±103 copies/50 ng RNA) and TNM2 (398±234 copies/50 ng RNA), although these values did not reach statistical significance through individual group sub-analysis, close to significant values were obtained when the low grade TNM1 stage was compared to higher TNM3 and TNM4 groups (TNM4 vs. TNM1, p=0.056; TNM3 vs. TNM1, p=0.061; TNM4 vs. TNM2, p=0.10; TNM3 vs. TNM2, p=0.10). With regard to the lymph node involvement, a decreased level of HuR transcript was seen in the node-positive tumours, 191±161 copies/50 ng RNA, p=0.34 compared to the level in node-negative tumours (28±115 copies/50 ng RNA) (Figure 3C).

According to the histological type, there was a decrease in the HuR transcripts in the lobular tumours (1.9±0.9 copies/50 ng RNA), compared to the ductal tumours (255±108 copies/50 ng RNA), the most common type of
breast cancer, although the value did not reach statistical significance \( (p=0.324) \). A relatively higher level of HuR transcripts was seen in the other types of breast cancer such as mucinous, medullary and tubular \((596\pm590 \text{ copies/50 ng RNA} , p=0.61)\) compared to the ductal tumours \((596\pm590 \text{ copies/50 ng RNA})\). As the sample numbers were very small, the statistical results between the other types of breast cancer were not convincing.

**HuR and ERs status.** In consideration of the oestrogen receptor (ER) status, no significantly different level of HuR transcript was revealed in the ER negative tumours, \((290\pm132 \text{ copies/50 ng RNA}, p=0.54)\) compared to the ER positive tumours \((166\pm155 \text{ copies/50 ng RNA})\) \((p=0.54)\). Similarly, there was no significant difference in the HuR transcript level of the ERβ negative tumours \((196\pm102 \text{ copies/50 ng RNA}, p=0.38)\) compared to that in the ERβ positive tumours \((461\pm272 \text{ copies/50 ng RNA})\) \((p=0.38)\).

**HuR and Nottingham Prognostic Index (NPI).** The prognostic potential of HuR expression was also examined in accordance with the NPI of the patients. The NPI-1 group \((\text{NPI score <3.4; n=59})\) represented patients with a good prognosis, NPI-2 group \((\text{NPI score 3.4-5.4; n=35})\) represented patients with a moderate prognosis, while the patients of NPI-3 group \((\text{NPI score >5.4; n=15})\) had a poor prognosis. The statistical analysis showed HuR expression was reduced in the patients with a moderate prognosis \((\text{NPI-2})\) \((242\pm232 \text{ copies/50 ng RNA})\) and poor prognosis \((\text{NPI-3})\) \((77.6\pm75 \text{ copies/50 ng RNA})\) compared with the patients with a good prognosis \((\text{NPI-1})\) \((283\pm115 \text{ copies/50 ng RNA})\), although these values did not reach statistical significance through individual group sub-analysis \((\text{NPI-2 vs. NPI-1, p=0.88; NPI-3 vs. NPI-1, p=0.14; Figure 3G})\).

**Prognostic relevance of HuR in breast cancer.** Regarding the clinical outcomes, the patients were divided into remaining disease free, with metastasis, with local recurrence, died of breast cancer and with bone metastasis groups, after a median 72 months follow-up. HuR transcript levels were found to be reduced in the latter four groups compared to levels observed in the patients remaining disease free group \((\text{Figure 4A})\). In particular, the patients with metastasis \((25\pm19 \text{ copies/50 ng RNA})\), those who died of breast cancer \((0.91\pm0.4 \text{ copies/50 ng RNA})\), and...
Figure 2. Immunohistochemical staining of HuR in human breast specimens. Left, HuR protein was well stained in the normal breast epithelial cells and absent from the stromal fibroblasts. Right, HuR protein was negative/weakly positive in the breast tumour cells.
RNA) or who had bone metastasis (35.9±20 copies/50 ng RNA) had significantly decreased levels of HuR transcripts, \( p=0.031 \), \( p=0.018 \) or \( p=0.038 \) respectively, in comparison with the patients who were disease free (303±125 copies/50 ng RNA). Therefore, the patients with poor prognosis (8.53±6.24 copies/50 ng RNA) including those with metastasis and who died of breast cancer had dramatically lower levels of HuR transcripts compared to the patients who were disease free (303±125 copies/50 ng RNA, \( p=0.021 \); Figure 4B).

\( \text{HuR expression correlated with prognosis and long term survival.} \) To determine whether the HuR transcript levels were associated with long-term survival, the patients were divided into those with high levels (n=69) and those with low levels (n=40) of HuR. The cut-off point was guided using the mean level of HuR in patients with moderate prognosis, namely, NPI-2 group (NPI 3.4-5.4, Figure 3G). As shown in the Kaplan–Meier survival curve (Figure 4C), high levels of HuR significantly correlated with higher overall survival (100% survival in patients with high levels of HuR vs. 79.3% survival in patients with low levels, \( p=0.06 \)). Further analysis (Figure 4D) showed that 90% of the patients with higher expression levels of HuR had disease-free survival compared to 74.1% of the patients who had low levels of HuR expression (\( p=0.28 \)) during the follow-up period.

Discussion

HuR displayed a strong distribution pattern in the three breast cancer cell lines examined. This is in agreement with a study focusing on breast infiltrating duct carcinoma and normal tissue counterparts, which revealed higher HuR expression and cytoplasmic presence in breast carcinoma (19). However, aberrant HuR levels were expressed within the breast tissue specimens with a decreased level of HuR transcript in the breast tumours compared to the normal background tissues, although the difference did not reach statistical significance. Consistent with the transcript level, the negative/ weakly positive immunohistochemical staining also suggested that HuR protein levels were reduced within the tumour tissues of the patients, compared to the intense epithelial staining of the normal breast tissues.
HuR expression in breast invasive ductal carcinoma has been reported to be associated with the clinical pathology and prognosis (20). In the present study, no obvious association of HuR transcript levels with breast cancer histological grade and type, ER or ERβ status was observed. However, the levels of HuR transcripts were lower in the more advanced TNM4 and TNM3, node-positive tumours, compared with the early stage TNM2 and TNM1, node-negative tumours, although these values did not reach statistical significance. Thus, there was no obvious correlation of HuR expression with the pathological status and clinical variables in the patients with breast cancer. This appears to be in contrast to the previous study, which showed by immunohistochemical staining that high cytoplasmic HuR expression was associated with poor histological differentiation, large tumour size, tumour aggression and poor patient survival rates in invasive ductal breast carcinoma (20).

The ER is one of the most important molecular factors used in clinical practice to predict the prognosis and response to therapy of breast cancer (21). The control of ER mRNA stability is mediated by HuR through binding to its 3'UTR and siRNA inhibition of HuR expression, which reduces both the steady-state and stability of ER mRNA (22). In the present study, there was no significant association of the level of HuR transcripts with ER status nor with ERβ status. HuR expression was reduced in the patients with a moderate NPI prognosis (NPI-2) or poor prognosis (NPI-3), compared with the patients with a good prognosis (NPI-1), although these values did not reach statistical significance (p=0.88, p=0.14 respectively).

The HuR mRNA levels were dramatically reduced in the patients with metastasis, those who died of breast cancer or those with bone metastasis, compared to the patients who were disease free (p=0.031, p=0.018 or p=0.038). Patients classed as poor prognosis including those with metastasis and who died of breast cancer displayed significantly lower levels of HuR transcripts compared to the patients who remained disease-free (p=0.021). Indeed, the patients with local recurrence displayed similar levels of HuR transcripts compared with the disease-free patients, although these values did not reach statistical significance (p=0.85). Finally, a correlation between HuR expression and overall survival

Figure 4. HuR transcript levels and clinical outcome. A, Disease status after median 72 months follow-up. B, Patients with poor prognosis, including metastasis and those who died of breast cancer, compared to the disease free patients. C, Kaplan–Meier survival curve indicating high levels of HuR transcripts (HuR(H)) correlated with longer overall survival, although the values did not reach statistical significance (p=0.06). D, High levels of HuR had similar disease free survival rates as samples containing lower HuR transcripts (HuR(L)) levels (p=0.28).
was demonstrated in the patients with breast cancer. High levels of HuR expression were associated with a greater overall survival, with close to significant values obtained (p=0.06). Thus, the Q-PCR-based studies, demonstrated a correlation between HuR expression and metastasis, death as a result of breast cancer, bone metastasis and overall survival in patients with breast cancer, suggesting that HuR acts as a prognostic indicator for breast cancer patients. Consistent with these findings, a previous report, examining the association HuR expression level with the degree of alteration of the bioenergetic phenotype of the tumour, showed that low tumour expression of HuR predicted a higher risk of disease recurrence in early stage breast cancer patients (23).

In summary, while no obvious association between the levels of HuR expression with pathological status of breast cancer was demonstrated, HuR may represent an independent prognostic factor in human breast cancer, as patients expressing high levels of HuR have a favourable prognosis. A prognostic role of HuR for breast cancer metastasis, particularly bone metastasis, is supported.

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