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ORIGINAL ARTICLE

Elevated Hu-Antigen Receptor (HuR) Expression is Associated with Tumor Aggressiveness and Poor Prognosis but not with COX-2 Expression in Invasive Breast Carcinoma Patients

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Abstract Hu-antigen R (HuR), a RNA-binding protein, is considered to play a crucial role in tumor development and progression by stabilizing or regulating a group of cellular mRNAs of cancer-related genes, such as cyclooxygenase-2 (COX-2). The present study aimed to evaluate the clinical significance of HuR and COX-2 expression in invasive breast carcinoma. HuR and COX-2 protein expression was assessed immunohistochemically on paraffin-embedded breast cancer tissue sections obtained from 121 patients and was statistically analyzed with clinicopathological parameters, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), as well as with tumor cells' proliferative capacity and overall and disease-free patients' survival. High HuR expression was positively associated with larger tumor size and advanced disease stage ($p = 0.0234$ and $p = 0.0361$, respectively), being more frequently observed in ER negative cases ($p = 0.0208$). High COX-2 expression was negatively associated with histological ($p < 0.0001$) and nuclear ($p = 0.0033$) grade and tumor cells' proliferative rate ($p = 0.0015$), being more frequently observed in luminal-A compared to other molecular subtypes ($p = 0.0221$). High HuR expression was associated with poor overall and disease-free patients' survival at both univariate (log-rank test, $p = 0.0092$ and $p = 0.0004$, respectively) and multivariate (Cox-regression analysis, $p = 0.0223$ and $p = 0.0004$, respectively) level. On the other hand, high COX-2 expression was associated with favorable overall and disease-free patients' survival merely at univariate level (log-rank test, $p = 0.0389$ and $p = 0.0154$, respectively). HuR expression was not associated with COX-2 expression (Spearman $R = 0.1489$, $p = 0.1032$). The present data support evidence that HuR is associated with tumor aggressiveness and poor prognosis in breast carcinoma, reinforcing its potential as promising therapeutic target in this type of neoplasia.

Keywords Hu-antigen R · COX-2 · Breast cancer · Immunohistochemistry · Clinicopathological parameters · Patients' prognosis

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Introduction

Hu-antigen R (HuR) or ELAV (embryonic lethal, abnormal vision, Drosophila)-like protein 1 (ELAVL1) belongs to the Hu/ELAV family and is an ubiquitously expressed RNA-binding post-transcriptional regulator [1]. HuR contains three highly conserved RNA binding domains that belong to the RNA recognition motif (RRM) superfamily [2]; RRM-1 and -2 bind to AU-rich elements (ARE), while RRM-3 binds to the mRNA poly(A) tail. A U-rich sequence approximately 17–20 nucleotides long, usually located within the 3′ untranslated

region (UTR) of the target mRNAs, has been identified as the RNA motif recognised by HuR [3]. HuR binds to this motif and regulates the stability, translation and nucleocytoplasmic translocation of target mRNAs. More specifically, HuR binding may stabilize the mRNA, indirectly increasing protein production [4], while its direct effect on translation efficiency can be either positive or negative [5, 6]. Moreover, mRNA polyadenylation, a procedure taking place in the nucleus, can also be modulated by HuR [7]. Additionally, HuR can be transported from the nucleus, where is most abundantly localized, to the cytoplasm, along with the bound mRNA [8] and this change in subcellular localization appears to be linked to the regulation of HuR function [9]. Notably, HuR can stabilize the mRNA of cyclooxygenase-2 (COX-2), an enzyme that catalyzes the synthesis of prostaglandins, and is associated with the promotion of carcinogenesis and tumor cell resistance to apoptosis [10, 11].

Breast cancer represents the most common malignancy and cause of cancer-related death, amongst women. Mammary tumors present highly complexity and heterogeneity, while global understanding of the underlined molecular mechanisms governing their origin and progression is still lucking [12]. Molecular imaging has been considered to exert a promising role in complementing and overcoming some of the limitations of traditional biomarkers by providing the ability to perform noninvasive, repeatable whole-body assessments [13]. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) define prognosis and identify tumors for targeted therapy, and remain the sole established single-molecule biomarkers defining the minimum breast cancer pathology data set [14]. ER-targeted endocrine therapies are effective for the treatment of patients with ER-positive breast tumors and tamoxifen is the most widely used endocrine anti-estrogen treatment. Interestingly, a number of studies has implicated HuR in ER and HER2 expression regulation and tamoxifen resistance, suggesting that HuR may play a crucial role in breast cancer development and possibly treatment, as recently reviewed by our group [15].

The most comprehensive studies so far have supported substantial evidence that HuR is implicated in several pathological conditions, such as atherosclerosis [16], tissue ischemia [17], pathologic inflammation [6] and neoplasia [15, 18, 19]. Notably, HuR has been suggested to participate in malignant transformation process by controlling cancer-relevant genes related with angiogenesis, differentiation, cell cycle, apoptosis, inflammatory response and cell signaling [15, 18–21]. Moreover, HuR appears to exert a critical role in tumor formation, growth and metastasis by binding to mRNA encoding proteins and by affecting their expression via mRNA stabilization and/or altered translation [15, 20, 21]. Enhanced HuR expression and cytoplasmic localization have been associated with malignant phenotype and poor patients' prognosis in several human malignancies [18, 22]. Interestingly, several studies have further suggested that HuR is implicated in malignant transformation of the breast, however, contradictory data have currently been reported regarding its clinical and prognostic impact in this type of neoplasia [23–31]. In view of the above considerations, the present study is aimed to evaluate the immunohistochemical expression of HuR and COX-2 in invasive breast carcinoma in association with multiple clinicopathological characteristics, tumor cells' proliferative capacity and ER, PR and HER2 expression, as well as overall and disease-free patients' survival.

Patients and Methods

Patients

One hundred twenty-one invasive breast carcinoma specimens obtained from an equal number of patients who underwent surgical resection due to breast cancer were included. The patients' age ranged from 33 to 85 years (mean 57 years). None of the patients had pre-operatively received radiation or chemotherapy. The institutional ethical committee of the Medical School of the University of Athens approved this study. Informed consent was signed by all patients in order to use for research purposes their biological samples and clinical data [32].

Haematoxylin and eosin staining was performed for routine histological examination. All cases were classified in accordance with World Health Organization criteria [32] and were classified as ductal or lobular. Nuclear grading was based on nuclear pleomorphism. Staging at the time of diagnosis was based on the TNM system [33]. The combined histological grade (1, 2 or 3) of infiltrating ductal and lobular breast carcinomas was obtained according to the modified Scarff-Bloom-Richardson histological system and the guidelines suggested by Nottingham City Hospital pathologists [34]. The clinicopathological characteristics of the series are shown in Table 1.

The patients were followed up for a time interval of 8 up to 210 months with a mean survival time of 81.44 ± 10.34 months. Overall survival was defined as the time interval between the date of surgery and the date of death due to breast carcinoma or the last follow-up. Disease-free survival was defined as the time interval between the date of surgery and the date of detection of recurrence or the date of last follow-up without recurrence for breast carcinoma. At the time of the last follow-up, 25 (20.7%) patients had died from disease, 15 (12.4%) were alive with disease and 81

Table 1 Associations of HuR and COX-2 expression with clinicopathological parameters in 121 invasive breast carcinoma patients

Clinicopathological parameters	HuR expression			COX-2 expression		
	Low (%)	High (%)	*p*-value	Low (%)	High (%)	*p*-value
N = 121	67 (55.4)	54 (44.6)		65 (53.7)	56 (46.3)	
Age (mean ± SD;ys)			0.9349			0.6534
≤ 57.0 ± 12.5 yrs	33 (27.3)	27 (22.3)		31 (25.6)	29 (24.0)	
> 57.0 ± 12.5 yrs	34 (28.1)	27 (22.3)		34 (28.1)	27 (22.3)	
Menopausal status			0.3860			0.5818
Premenopausal	21 (17.4)	21 (17.4)		24 (19.8)	18 (14.9)	
Postmenopausal	46 (38.0)	33 (27.3)		41 (33.9)	38 (36.4)	
Histopathological type			0.6330			0.3583
Ductal	47 (38.8)	40 (33.1)		49 (40.5)	38 (31.4)	
Lobular	20 (16.5)	14 (11.6)		16 (13.2)	18 (14.9)	
Histological Grade			0.0921			**<0.0001**
1 + 2	46 (38.0)	29 (24.0)		27 (22.3)	48 (39.7)	
3	21 (17.4)	25 (20.7)		38 (31.4)	8 (6.6)	
Nuclear Grade			0.0606			**0.0033**
1	35 (28.9)	19 (15.7)		21 (17.4)	33 (27.3)	
2 + 3	32 (26.5)	35 (28.9)		44 (36.4)	23 (19.0)	
Molecular subtype			0.0793			**0.0221**
Luminal-A	24 (19.8)	20 (16.5)		16 (13.2)	28 (23.1)	
Luminal-B	28 (23.1)	12 (9.9)		26 (21.5)	14 (11.6)	
HER2	5 (4.1)	7 (5.8)		9 (7.4)	3 (2.5)	
Triple negative	10 (8.3)	15 (12.4)		14 (11.6)	11 (9.1)	
Tumor size			**0.0234**			0.0535
< 2 cm	25 (20.7)	10 (8.3)		14 (11.6)	21 (17.4)	
≥ 2 cm	42 (34.7)	44 (36.4)		51 (42.1)	35 (28.9)	
Lymph nodes			0.4650			0.4463
Non infiltrated	33 (27.3)	23 (19.0)		28 (23.1)	28 (23.1)	
Infiltrated	34 (28.1)	31 (25.6)		37 (30.6)	28 (23.1)	
Histopathological stage			**0.0361**			0.1465
I	22 (18.2)	7 (5.8)		11 (9.1)	18 (14.9)	
II	36 (29.8)	36 (29.8)		42 (34.7)	30 (24.8)	
III + IV	9 (7.4)	11 (9.1)		12 (9.9)	8 (6.6)	
ER expression			**0.0208**			0.0892
Negative	22 (18.2)	29 (24.0)		32 (26.4)	19 (15.7)	
Positive	45 (37.2)	25 (20.7)		33 (27.3)	37 (30.6)	
PR expression			0.2384			0.9327
Negative	30 (24.8)	30 (24.8)		32 (26.4)	28 (23.1)	
Positive	37 (30.6)	24 (19.8)		33 (27.3)	28 (23.1)	
HER2 expression			0.7574			0.1615
Negative	57 (47.1)	47 (38.8)		52 (43.0)	50 (41.3)	
Positive	10 (8.3)	7 (5.8)		13 (10.7)	6 (5.0)	
Ki-67 protein statement			0.8065			**0.0015**
Below median value	32 (26.5)	27 (22.3)		23 (19.0)	36 (29.8)	
Over median value	35 (28.9)	27 (22.3)		42 (34.7)	20 (16.5)	

Statistically significant *p*-values are depicted by bold

(66.9%) were alive and disease-free. All patients received conventional postoperative treatment depending on the extent of the disease, including adjuvant chemotherapy, radiation therapy and anti-estrogen therapy, when indicated, according to the consensus recommendations at the time [35].

Immunohistochemistry

Commercially available rabbit polyclonal anti-HuR (H-280, sc-20,694) and anti-COX-2 (H-62, sc-7951) IgG antibodies (Santa Cruz Biochemicals, Santa Cruz, CA, USA) were used for HuR and COX-2 immunostainings on formalin-fixed, paraffin-embedded breast tissue sections. Four μm thick tissue sections were deparaffinized, rehydrated, immersed in 3% H_2O_2 for 30 min and microwaved at 750 W in 0.01 M citrate buffer (pH 6.0) for 15 min and then they left to cool down in TBS. Incubation with primary HuR and COX-2 antibodies was performed for 1 h at room temperature (37 °C), at a dilution 1:100 and 1:200, respectively. The standard two-step peroxidase conjugated polymer technique (DAKO Envision kit, DAKO, Carpinteria, CA, USA) was then performed. At a next step, immunostainings were visualized with diaminobenzidine tetrahydrochloride solution (DAB; Sigma, Saint Louis, MO, USA). Sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Appropriate negative controls were performed by omitting the primary HuR and COX-2 antibodies and/or substituting them with an irrelevant anti-serum. Lung and thyroid cancer tissue sections with known enhanced HuR and COX-2 expression were used as positive control [19, 36]. A mouse anti-human Ki-67 antigen; IgG1k antibody (clone MIB-1, Dakopatts, Glostrup, Denmark) were used to evaluate the tumor cells' proliferative capacity [36, 37]. The expression of ER, PR and HER2 was assessed immunohistochemically, as previously described [38].

Evaluation of Immunohistochemistry

Immunohistochemical evaluation was performed by counting at least 1000 tumor cells in each case by two independent observers blinded to the clinical data. Specimens were considered HuR and COX-2 -positive when more than 5% of tumor cells within the section were positively stained. HuR and COX-2 immunoreactivity was scored according to the percentage of positive tumor cells as 0: negative staining- 0-4% of tumor cells positive; 1: 5–24% of tumor cells positive; 2: 25–49% of tumor cells positive; 3: 50–100% of tumor cells positive, and its intensity as 0: negative staining, 1: mild staining; 2: intermediate staining; 3: intense staining. Finally, HuR and COX-2 expression was classified as low; if the total score was 0 or 2 and high; if the total score was ≥3. In this way, we ensure that each group has a more homogeneous and sufficient number of cases in order to be comparable with the other groups [19, 36, 37, 39].

Staining for ER and PR was evaluated according to CAP/ASCO recommendations, i.e. ER and PR assays are considered positive if there are at least 1% positive tumor nuclei in the sample in the presence of the expected reactivity of internal and external controls [40]. The fraction of HER2 positive stained cells was scored according to CAP/ASCO guidelines [41]. Ki-67 immunoreactivity was classified according to the percentage of positively stained breast cancer cells exceeded the median percentage value into two categories (below and over mean value), as previously reported [19, 36, 37, 39].

Statistical Analysis

The associations of HuR and COX-2 protein expression with clinicopathological variables, tumor cells' proliferative capacity and ER, PR and HER2 protein expression were evaluated by chi-square test. The Kaplan-Meier method was applied to construct survival curves and the log rank test was applied to compare the differences between the curves. To assess, at a multivariate level, the associations between the potential prognostic marker and overall and disease-free patients' survival, a Cox proportional-hazard regression model was developed. A p-value lower than 0.05 was considered as the limit of statistical significance. For all analyses SPSS for Windows Software was used (SPSS Inc., 2003, Chicago, USA).

Results

Associations of HuR Expression with Clinicopathological Parameters and Patients' Survival

HuR positivity (IHC score > 0) was noted in 104 (86.0%) out of 121 breast cancer cases. The intensity of HuR immunostaining was mild in 39 (37.5%), moderate in 42 (40.4%) and intense in 23 (22.1%) out of 104 HuR-positive breast carcinoma cases. Fifty-four (44.6%) out of the 121 examined cases presented high HuR expression (IHC score ≥ 3). The subcellular pattern of HuR distribution was cytoplasmic in 102 (84.3%) and both cytoplasmic and nuclear in 19 (15.7%) out of the 121 examined cases. Normal surrounding areas adjacent to tumor were found either negative or presented mild nuclear immunostaining for HuR. Representative HuR cytoplasmic or cytoplasmic and nuclear immunostainings are depicted in Fig. 1a and b, respectively. Seventy (57.9%) out of 121 breast carcinoma cases were ER positive. PR positivity was noted in 61 (50.4%) out of 121 breast carcinoma cases, while 17 (14.1%) cases were HER2 positive. Sixty-five (53.7%) out of 121 breast carcinoma cases were classified as luminal-A, 17 (14.1%) cases as luminal-B, 25 (20.7%) case as triple negative and 14 (11.6%) as HER2 phenotype.

In cross-tabulation, high HuR expression was significantly associated with larger tumor size and advanced disease stage (Table 1, $p = 0.0234$ and $p = 0.0361$, respectively). High HuR expression was significantly more frequently observed in ER negative breast carcinoma cases (Table 1, $p = 0.0208$). Borderline associations between high HuR expression and

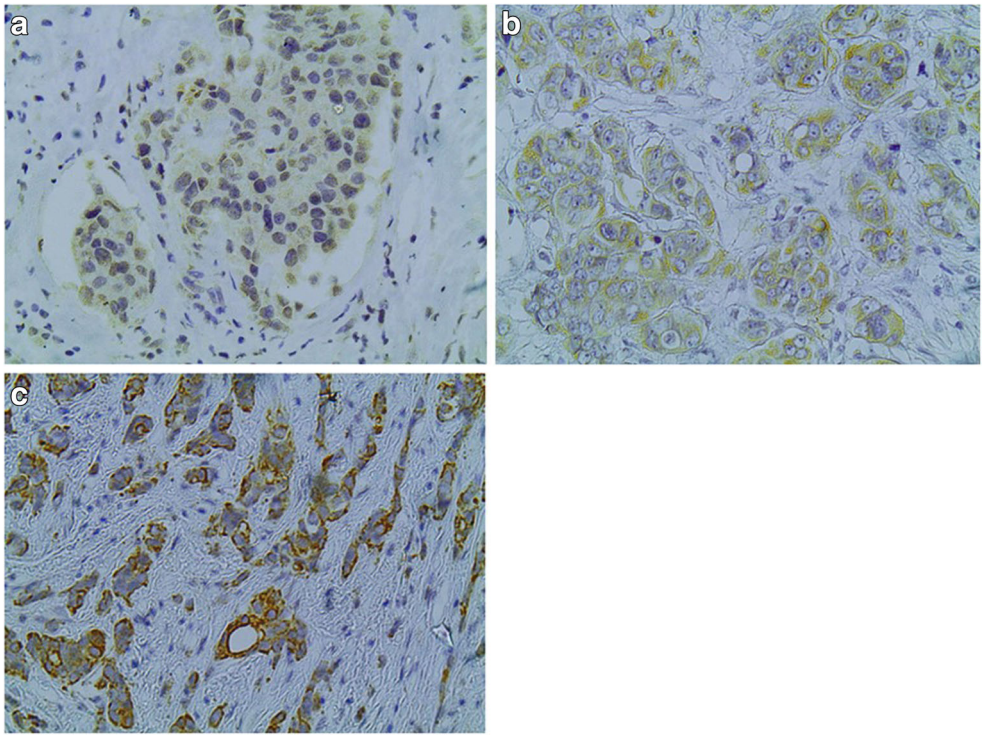

Fig. 1 Representative immunostainings for **a**. Cytoplasmic and nuclear HuR expression (X400) **b**. Cytoplasmic HuR expression (X400) and **c**. Cytoplasmic COX-2 expression (X400). Streptavidin-biotin-peroxidase, DAB chromogen, Harris hematoxylin counterstain

histological and nuclear grade of differentiation were recorded (Table 1, $p = 0.0921$ and $p = 0.0606$, respectively). High HuR expression was more frequently observed in PR negative breast carcinoma cases, at a no significant level though (Table 1, $p = 0.2384$). Triple negative and HER2 subtype breast carcinoma cases presented an increased incidence of high HuR expression compared to luminal-A and luminal-B molecular subtypes (Table 1, $p = 0.0793$). Cytoplasmic subcellular HuR distribution was significantly more frequently observed in postmenopausal breast carcinoma patients ($p = 0.0207$), as well as in those presenting tumor infiltrated lymph nodes ($p = 0.0350$). Subcellular HuR distribution was not associated with either any of the other clinicopathological parameters examined or patients' survival (data not shown).

Kaplan-Meier survival curves indicated that breast carcinoma patients presenting high HuR expression showed significantly shorter overall survival times compared to those with low HuR expression (Fig. 2a, log-rank test, $p = 0.0092$). In multivariate analysis, histological type and grade, tumor size, HER2 expression, Ki-67 protein statement and HuR expression were identified as independent prognostic factors of overall patients' survival (Table 2, Cox-regression analysis, $p = 0.0223$, $p = 0.0495$, $p = 0.0316$, $p = 0.0272$, $p < 0.0001$ and $p = 0.0184$, respectively). Kaplan-Meier survival curves indicated that breast carcinoma patients presenting high HuR expression showed significantly shorter disease-free survival times compared to those with low HuR expression (Fig. 2b, log-rank test, $p = 0.0002$). In multivariate analysis, histological type and grade, tumor size, HER2 expression, Ki-67 protein statement and HuR expression were identified as independent prognostic factors of disease-free patients' survival (Table 3, Cox-regression analysis, $p = 0.0057$, $p = 0.0434$, $p = 0.0326$, $p = 0.0018$, $p = 0.0001$ and $p = 0.0004$, respectively).

Associations of COX-2 Expression with Clinicopathological Parameters and Patients' Survival

COX-2 positivity (IHC score > 0) was noted in 93 (76.9%) out of 121 breast cancer cases. The intensity of COX-2 immunostaining was mild in 17 (18.3%), moderate in 38 (40.9%) and intense in 38 (40.9%) out of 93 COX-2 positive breast carcinoma cases. Fifty-six (46.3%) out of the 121 examined cases presented high COX-2 expression (IHC score ≥ 3). The subcellular pattern of COX distribution was cytoplasmic in all the examined cases. Normal surrounding areas adjacent to tumor were found either negative or presented mild cytoplasmic immunostaining for COX-2. Representative COX-2 immunostaining is depicted in Fig. 1c.

In cross-tabulation, high COX-2 expression was negatively associated with histological and nuclear grade (Table 1, $p < 0.0001$ and $p = 0.0033$, respectively). Luminal-A molecular subtype breast carcinoma cases presented a significantly increased incidence of high COX-2 expression compared to luminal-B, triple negative and HER2 subtypes (Table 1, $p = 0.0221$). High COX-2 expression was marginally associated with lower tumor size (Table 1, $p = 0.0535$). High COX-2

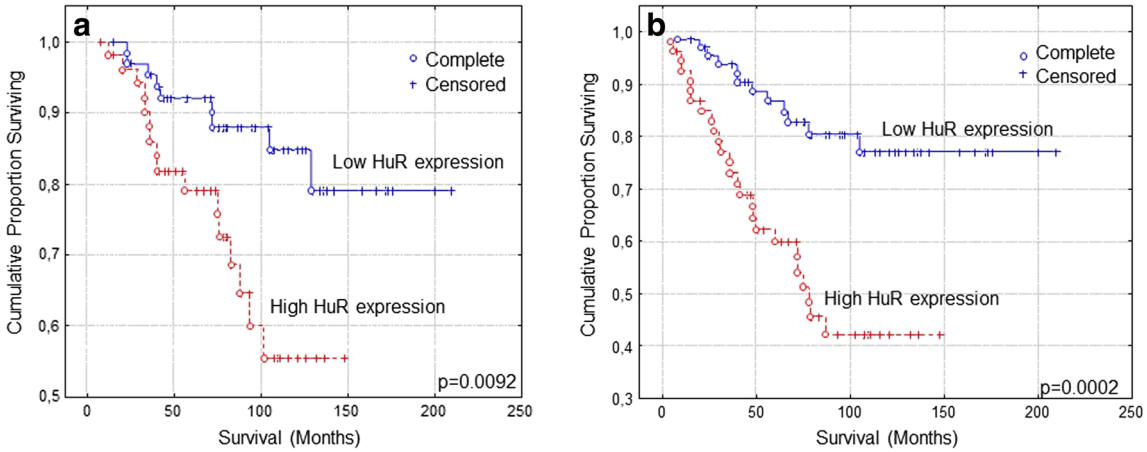

Fig. 2 Kaplan-Meier survival analysis stratified according to HuR expression in 121 breast carcinoma patients for: **a**. Overall patients' survival and **b**. Disease-free patients' survival

expression was significantly negatively associated with tumor cells' proliferative rate (Table 1, $p = 0.0015$), presenting also a trend of positive association with ER expression (Table 1, $p = 0.0892$). Spearman rank order correlation analysis revealed that HuR expression was not associated with COX-2 expression (Spearman $R = 0.1489$, $p = 0.1032$). HuR subcellular distribution was not also correlated with COX-2 expression ($p = 0.3689$).

Kaplan-Meier survival curves indicated that breast carcinoma patients presenting high COX-2 expression showed significantly longer overall survival times compared to those with low COX-2 expression (Fig. 3a, log-rank test, $p = 0.0389$). In multivariate analysis, histological type and grade, tumor size and Ki-67 protein statement but not COX-2 expression were identified as independent prognostic factors of overall patients' survival (Table 4, Cox-regression analysis, $p = 0.0104$, $p = 0.0212$, $p = 0.0073$, $p = 0.0008$ and $p = 0.3683$, respectively). Kaplan-Meier survival curves also indicated that breast carcinoma patients presenting high COX-2 expression showed significantly longer disease-free survival times compared to those with low COX-2 expression (Fig. 3b, log-rank test, $p = 0.0154$). In multivariate analysis, histological type and grade, tumor size and Ki-67 protein statement but not COX-2 expression were identified as independent prognostic factors of overall patients' survival (Table 5, Cox-regression analysis, $p = 0.0047$, $p = 0.0194$, $p = 0.0022$, $p = 0.0026$ and $p = 0.0836$, respectively).

Discussion

A gradually increasing number of studies have currently documented that HuR overexpression and cytoplasmic localization are associated with crucial clinicopathological parameters for patients' management and prognosis in several types of human malignancy, as recently reviewed by our group [15]. Moreover, a number of clinical studies in breast cancer patients have demonstrated that elevated HuR expression was correlated with crucial clinicopathological parameters and patients' survival, indicating that high HuR expression levels may constitute an aggravating factor for tumor growth and metastasis [15]. However, as far as concern breast carcinoma, opposite data also exist, supporting the notion that low HuR expression levels are associated with tumor aggressiveness and poor prognosis [15]. Moreover, a lot of currently available data supported substantial evidence that COX-2 is associated with the promotion of carcinogenesis and tumor cell resistance to apoptosis [10, 11].

Table 2 Multivariate analysis for HuR expression and overall patients' survival

Clinicopathological variables	Overall survival	
	HR (95% CI)	*p*-value
Histological type (Ductal / Lobular)	0.266 (0.019–0.583)	**0.0223**
Histological grade (I + II / III)	0.423 (0.122–1.034)	**0.0495**
Tumor size (< 2 cm / > 2 cm)	9.900 (4.132–18.950)	**0.0316**
HER-2 expression (Negative / Positive)	3.193 (1.383–6.785)	**0.0272**
Ki-67 statement (Below / Over median value)	9.489 (6.731–12.549)	**<0.0001**
HuR expression (Low / High)	2.948 (1.294–4.789)	**0.0184**

Statistically significant *p*-values are depicted by bold

Table 3 Multivariate analysis for HuR expression and disease-free patients' survival

Clinicopathological variables	Disease-free survival	
	HR (95% CI)	*p*-value
Histological type (Ductal / Lobular)	0.280 (0.034–0.598)	**0.0057**
Histological grade (I + II / III)	0.489 (0.138–1.143)	**0.0434**
Tumor size (< 2 cm / > 2 cm)	3.359 (1.726–7.420)	**0.0327**
HER-2 expression (Negative / Positive)	3.802 (1.542–7.329)	**0.0018**
Ki-67 statement (Below / Over median value)	4.527 (3.354–6.781)	**0.0001**
HuR expression (Low / High)	3.714 (2.443–5.140)	**0.0004**

Statistically significant *p*-values are depicted by bold

In this aspect, the present study is aimed to assess the expression levels of HuR and COX-2 in breast carcinoma in order to further clarify their clinical and prognostic impact in this type of neoplasia. According to our results, approximately half of the examined cases presented high HuR expression levels and all the examined cases presented negative or mild nuclear HuR immunostaining in non-malignant breast tissue. Moreover, it should be noted that HuR subcellular distribution was found predominately cytoplasmic in the vast majority of the examined breast carcinoma cases, which suggested that HuR may be translocated from nucleus to cytoplasm during the malignant breast transformation process.

The present study also showed that high HuR expression was associated with larger tumor size and advanced disease stage. High HuR expression was also more frequently observed in ER negative breast carcinoma cases. Borderline associations between high HuR expression and histological and nuclear grade of differentiation were also recorded, while triple negative and HER2-type breast cancer cases presented an increased incidence of high HuR expression compared to luminal-A and -B molecular subtypes. Moreover, cytoplasmic subcellular HuR distribution was more frequently observed in postmenopausal breast carcinoma patients, as well as in those presenting tumor infiltrated lymph nodes. These findings supported substantial evidence for a potential crucial role of HuR in breast malignant progression that affect patients' survival. The strong association found between high HuR expression and poor overall and disease-free patients' survival further suggested that HuR may represent a potential negative prognosticator in invasive breast carcinoma. These data also reinforce the therapeutic utility of HuR targeting in breast cancer chemoprevention, since HuR appears to be a common denominator and regulator for a number of molecular pathways crucial for tumor formation, growth and metastasis, being implicated in chemoresistance mechanisms to therapeutic drugs, such as tamoxifen, as well as being associated with important potential therapeutic targets, such as cyclin D1, CDK1, CDK7, MPP-13 and YES1 [15].

In addition, our results are in accordance to several previously published studies [23–31]. In fact, a clinical study conducted on 97 ductal breast carcinoma patients revealed significant associations between elevated total HuR expression (calculated from the combination of staining intensity and extent of positivity in tumor cells) and advanced tumor histological grade and HER2-negative status [23]. Furthermore, cytoplasmic HuR expression pattern (calculated from the combination of staining intensity and extent of positivity in tumor cells) was positively associated with histological grade in 208

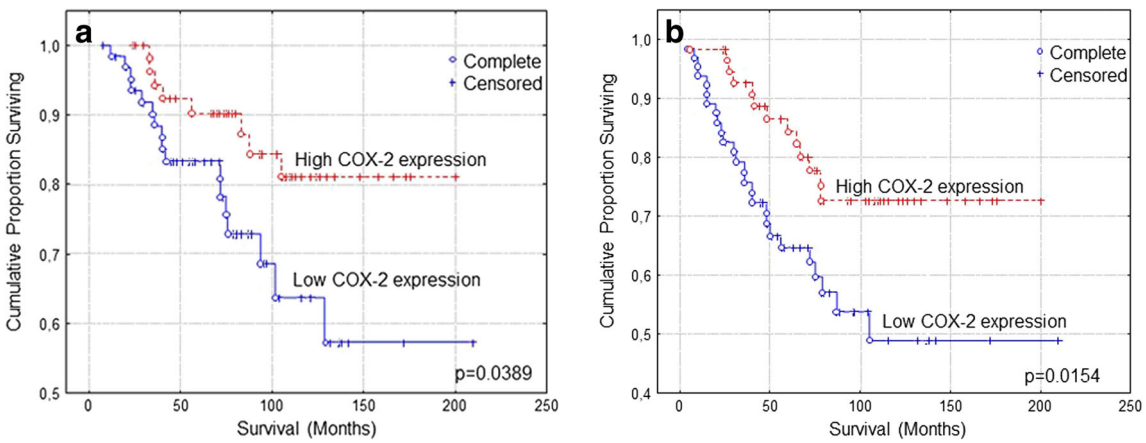

Fig. 3 Kaplan-Meier survival analysis stratified according to COX-2 expression in 121 breast carcinoma patients for: **a**. Overall patients' survival and **b**. Disease-free patients' survival

Table 4 Multivariate analysis for COX-2 expression and overall patients' survival

Clinicopathological variables	Overall survival	
	HR (95% CI)	*p*-value
Histological type (Ductal / Lobular)	0.221 (0.021–0.597)	**0.0104**
Histological grade (I + II / III)	0.344 (0.101–0.934)	**0.0212**
Tumor size (< 2 cm / > 2 cm)	16.568 (7.152–24.302)	**0.0073**
HER-2 expression (Negative / Positive)	1.980 (0.583–6.673)	0.1610
Ki-67 statement (Below / Over median value)	6.843 (3.451–12.769)	**0.0008**
COX-2 expression (Low / High)	0.663 (0.098–3.559)	0.3683

Statistically significant *p*-values are depicted by bold

invasive breast carcinoma patients [24]. Enhanced cytoplasmic HuR staining intensity was correlated with advanced patients' age and tumor histological grade in 82 breast carcinoma patients [26], with increased tumor grade and larger tumor size in another three studies conducted on 133, 208 and 139 invasive breast carcinoma patients, respectively [24, 27, 28], as well as with increased histological grade and ductal tumor type in 525 familial non-BRCA1/2 cases [29], and in 76 invasive breast carcinoma patients receiving paclitaxel and anthracycline-based neoadjuvant chemotherapy [25]. In accordance with the present findings, enhanced HuR staining intensity was correlated with ER-negative status in familial non-BRCA1/2 cases [29] and in invasive carcinoma patients receiving paclitaxel and anthracycline-based neoadjuvant chemotherapy [27]. Moreover, enhanced HuR staining intensity was correlated with PR-negative status in ductal in situ breast carcinoma [25], in familial non-BRCA1/2 cases [29], and in invasive breast carcinoma patients receiving paclitaxel and anthracycline-based neoadjuvant chemotherapy [27]. In contrast, elevated cytoplasmic HuR staining intensity was associated with PR-, ER- and HER2-positive status in a more recent study conducted by Zhu et al. [26].

Regarding the role of HuR as a prognosticator in breast cancer patients and in accordance with our findings, enhanced cytoplasmic HuR protein expression was identified as an independent prognostic factor for shorter overall and/or disease-free survival rate in ductal invasive breast carcinoma patients [26, 28], as also in invasive breast carcinoma patients receiving paclitaxel and anthracycline-based neoadjuvant chemotherapy [27], and in familial non-BRCA1/2 patients [29]. In contrast, other studies documented that low HuR mRNA expression was associated with poor prognosis and increased risk of disease recurrence in ductal invasive breast carcinoma patients [30, 31]. To this point, it should be noted that the first four studies that are in accordance with our findings applied an immunohistochemical technique to quantify HuR expression at protein level, whereas the last two studies that found an opposite association between HuR expression and patients' prognosis quantified HuR expression at mRNA level by the use of reversed transcription polymerase chain reaction technique. In this aspect, the above controversy may be ascribed to potential post-transcriptional modifications of HuR protein that may affect its clinical and prognostic impact. Different antibodies used may also be responsible for this controversy.

The present study also revealed that high COX-2 expression was negatively associated with histological and nuclear grade, and tumor cells' proliferative rate, being more frequently observed in luminal-A compared to other molecular subtypes. High COX-2 expression was also associated with favorable patients' prognosis. These findings are in accordance with previous data [42]. Moreover, COX-2 expression was inversely associated with tumor size, disease stage and HER-2 expression and positively with ER expression, at a non-significant level though. These findings supported substantial evidence for a potential crucial role of COX-2 in breast malignant progression that affect patients' survival. Denkert

Table 5 Multivariate analysis for COX-2 expression and disease-free patients' survival

Clinicopathological variables	Disease-free survival	
	HR (95% CI)	*p*-value
Histological type (Ductal / Lobular)	0.267 (0.032–0.531)	**0.0047**
Histological grade (I + II / III)	0.423 (0.122–1.018)	**0.0194**
Tumor size (< 2 cm / > 2 cm)	5.427 (2.228–8.950)	**0.0022**
HER-2 expression (Negative / Positive)	2.140 (0.598–5.229)	0.0522
Ki-67 statement (Below / Over median value)	3.118 (1.564–5.891)	**0.0026**
COX-2 expression (Low / High)	0.536 (0.098–2.320)	0.0836

Statistically significant *p*-values are depicted by bold

et al. also reported that elevated HuR expression was associated with increased COX-2 expression in human breast carcinoma [24]. In contrast, we did not find any association between HuR and COX-2 expression in our cohort study, while not any other existing study has reported such an association as far as concern breast carcinoma.

Conclusions

The present study supported clinical evidence that elevated HuR expression is associated with advanced tumor aggressiveness and poor prognosis in patients with invasive breast carcinoma. Moreover, HuR translocation from nucleus to cytoplasm was observed, which may suggest that this is a potential event during malignant breast transformation process. On the other hand, COX-2 expression was associated with less tumor aggressiveness and favorable prognosis in invasive breast carcinoma patients. Additional research conducted on larger cohorts and on each molecular subtype separately should be performed in order to explore the exact molecular pathways in which HuR and COX-2 are implicated, as well as to evaluate their role in drug resistance.

Compliance with Ethical Standards

Conflict of Interest All authors verify that they have not accepted any funding or support from an organization that may in any way gain or lose financially from the results of the present study. All authors verify that they have not been employed by an organization that may in any way gain or lose financially from the results of the present study. None authors have any other conflicting interest.

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