Prognostic Role of HuR in Hereditary Breast Cancer

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Abstract

Purpose: HuR is an mRNA-binding protein that enhances the stability of certain transcripts and can regulate their translation. Elevated cytoplasmic expression of HuR protein has been linked to carcinogenesis and is associated with reduced survival in breast, ovarian, and gastric adenocarcinomas.

Experimental Design: Here, we have explored the relevance of HuR in familial breast cancer. Tumor samples were collected from patients with identified *BRCA1* ($n = 51$) or *BRCA2* ($n = 47$) mutations or familial non-*BRCA1/2* cases ($n = 525$), and analyzed by immunohistochemistry.

Results: Among familial non-*BRCA1/2* breast cancer patients, cytoplasmic HuR protein expression was present in 39.4% of the cases and was associated with estrogen receptor negativity, progesterone receptor negativity, p53 positivity, high tumor grade, and ductal type of the tumor. In multivariate analysis, cytoplasmic HuR expression was an independent marker of reduced survival in the non-*BRCA1/2* group along with tumor size >2 cm, lymph node metastasis, and high histologic grade. In patients with *BRCA1* or *BRCA2* mutations, cytoplasmic HuR expression was more frequent (62.7% for *BRCA1* and 61.7% for *BRCA2*) than in the non-*BRCA1/2* group, but in *BRCA*-mutated subgroups cytoplasmic HuR expression did not associate with survival.

Conclusions: Our results show that HuR is an important prognostic factor in familial breast cancer patients and may contribute to carcinogenesis in this disease.

Deregulation of gene expression is a hallmark of carcinogenic process. Gene expression is regulated by a series of events that can take place at both transcriptional and posttranscriptional levels. Posttranscriptional regulation of mRNA turnover is an important process in the control of eukaryotic gene expression (1, 2). HuR (or HuA) is a ubiquitously expressed RNA-binding factor related to *Drosophila* embryonic lethal abnormal vision protein (3). It is a member of the Hu family, and the other three members, HuB/HelN1, HuC, and HuD, are primarily expressed in the neuronal tissues. In unstimulated conditions, HuR is primarily localized to the nucleus but it can shuttle between the nucleus and the cytoplasm. In the nucleus, HuR binds to a U-rich motif in the target mRNA and also to certain protein partners, and this complex is then transported to the cytoplasm where HuR can stabilize the transcript and/or regulate its translation (4, 5). Activation of the mitogen-activated protein kinases increase the cytoplasmic content of HuR, which leads to enhanced stability of its target transcripts (6, 7), such as many oncogenic gene products (1, 2).

The first link of Hu proteins to carcinogenesis was found in small-cell lung cancer patients, in whom they can act as autoantigens and evoke an immune response against neuronal tissues (8, 9). Elevated HuR expression has been found in high-grade brain tumors (10) and in chemically induced lung tumors in mice (11). The first indication of elevated HuR expression in a human adenocarcinoma was seen in colorectal cancer (12). Lopez de Silanes et al. (13) showed that cytoplasmic HuR expression is elevated in a wide variety of human carcinomas when compared with normal tissue samples, which was especially evident in colon cancer. However, cytoplasmic or nuclear HuR immunopositivity did not show any prognostic value in colorectal cancers (14). In contrast, the importance of HuR expression in human malignancies has been emphasized by data that show cytoplasmic HuR expression to be a marker of poor prognosis in breast, ovarian, and gastric adenocarcinomas (15–18).

Family history is one of the strongest risk factors for breast cancer development, and ~7% of newly diagnosed cases are due to hereditary predisposition (19). Two tumor-suppressor genes, *BRCA1* and *BRCA2*, have been identified and shown to predispose to breast and ovarian cancer (20, 21). Both *BRCA* genes participate in DNA repair by homologous recombination, and, in addition, *BRCA1* can regulate transcription and cell cycle (22, 23). Despite their shared function in DNA repair, BRCA1 and BRCA2 proteins probably also perform distinct functions in biological processes, which could explain

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differences in the clinicopathologic variables in patients carrying mutations in these genes. Here, we have explored the association of HuR with clinicopathologic variables and its prognostic significance in hereditary breast cancer.

Materials and Methods

Patients. The study material included tumor specimens from women with family history of breast cancer, who were treated for primary breast adenocarcinoma at the Helsinki University Central Hospital (24). The patients' *BRCA1/2* gene mutation status was determined by screening the entire coding regions and exon-intron boundaries using the protein truncation test and denaturing gradient gel electrophoresis, as previously described (25, 26). We were able to retrieve survival data and immunohistochemical scores from 641 invasive cases (from a total of 706 cases) of which 525 patients were *BRCA1/2* negative and 98 patients were *BRCA1/2* mutation carriers. For 18 patients, the *BRCA1/2* gene mutation status was unknown, and these samples were excluded from the analysis. Two hundred twenty-four of the analyzed familial non-*BRCA1/2* tumors came from patients with a stronger family history (at least three first- or second-degree relatives with breast or ovarian cancer, including the proband), and 301 from patents with two affected first-degree relatives (including the proband). The genealogies were confirmed through population registries and cancer diagnosis from the Finnish Cancer Registry. The median age at the time of diagnosis was 55 years (range 22-96 years), and the median duration of the follow-up of the patients still alive was 5.4 years (range 1.0-9.4 years) and of all patients 5.2 years (range 0.2-9.4 years). Of non-*BRCA1/2* patients, 83.2% were operated (43.9% with mastectomy and 39.3% with resection), 76.2% received postoperative radiation therapy, 30.3% received adjuvant chemotherapy, and 36.1% received endocrine therapy. Of *BRCA1/2* mutation carriers, 80.4% were operated (43.1% with mastectomy and 37.3% with resection), 73.2% received postoperative radiation, 39.0% received adjuvant chemotherapy, and 14.6% received endocrine therapy.

Immunohistochemistry. HuR protein expression was analyzed from tumor tissue microarrays using immunohistochemical staining. From each formalin-fixed and paraffin-embedded breast cancer specimen, four cores (diameter 0.6 mm) of the most representative area were used for the preparation of tumor tissue microarray blocks as described previously (27). Sections of 5 μm were cut and processed for immunohistochemistry. The tissue microarray slides were stained with a mouse monoclonal anti-human HuR antibody (19F12; a kind gift from Henry Furneaux, University of Connecticut Health Center, Farmington, CT) at a dilution of 1:10,000 (1.0 μg/mL). The immunostaining protocol for HuR was carried out as described previously (15). HuR immunoreactivity was scored from the familial breast cancer specimens based on the following criteria: nuclear staining only, low intensity of cytoplasmic HuR staining present (visible with ×100 or a higher magnification), and high intensity of cytoplasmic HuR staining present (visible with ×50 or a lower magnification). HuR staining set contained two predetermined colon carcinoma control slides, one of which contained only nuclear staining in the tumor cells and another one with cytoplasmic immunopositivity. Statistical analyses were done according to HuR cytoplasmic positivity versus negativity. Nuclear positivity was seen in 90.9% of the cases and an additional cytoplasmic staining in 42.4% of the cases, and only cytoplasmic staining in 0.6% of the cases. Cytoplasmic HuR staining was considered positive if any of the four core samples showed positivity. Within an individual core, the staining pattern was relatively uniform. The samples were blinded and scored independently by M.H. and A.R. In a case of discordant scores, the specimens were reevaluated using a multiheaded microscope and the consensus score was used for further analyses.

Statistical analysis. The association between HuR staining and clinicopathologic variables and prognostic variables was assessed using the χ^2 test. Life tables were computed according to the Kaplan-Meier method, and survival curves were compared with the log-rank test. Multivariate survival analysis was done with the Cox proportional hazards model using the following covariates: HuR expression, tumor size (T_1 versus T_2-T_4), lymph node status, histologic grade, estrogen receptor and progesterone receptor status, and p53 immunostaining. Cox regression was done using a backward stepwise selection of variables, and a P value of 0.05 was adopted as the limit for inclusion of a covariate. The backward stepwise algorithm was used to pick the best combination of prognostic factors to explain the mortality in the study population. Hazard ratios are provided for each covariate. The data were analyzed by using SPSS for Windows v12.0.1 (SPSS, Inc.). The study was done with the informed consent of patients as well as permissions from the ethics committee of the Helsinki University Central Hospital and from the Ministry of Social Affairs and Health in Finland.

Results

HuR expression and its association with clinicopathologic variables in familial breast cancer. Cytoplasmic HuR immunoreactivity was less frequent in non-*BRCA1/2* cases (39.4%, 207 of 525) when compared with either *BRCA1* mutation carriers (62.7%, 32 of 51; $P = 0.016$) or *BRCA2* mutation carriers (61.7%, 29 of 47; $P = 0.0049$). The cytoplasmic expression of HuR was similar between tumors from non-*BRCA1/2* families with only two affected individuals (36.6%, 82 of 224) and tumors from larger breast cancer families (41.5%, 125 of 301; $P = 0.2538$), and further analyses were carried out in the whole material. Cytoplasmic HuR expression in familial non-*BRCA1/2* cases was associated with negative estrogen receptor staining ($P < 0.0001$), negative progesterone receptor status ($P = 0.0003$), p53 immunopositivity ($P < 0.0001$), high histologic grade ($P = 0.0001$), and ductal type of the tumor ($P = 0.0216$; Table 1). In the *BRCA1* mutation carrier group ($n = 51$), the cytoplasmic HuR expression was associated with positive staining for p53 ($P = 0.0025$). In the *BRCA2* group ($n = 47$), we found no association between the cytoplasmic HuR expression and clinicopathologic variables, although there was a trend regarding p53 positivity ($P = 0.0560$). In fact, all p53-positive specimens were also positive for cytoplasmic HuR in both *BRCA1* ($n = 12$) and *BRCA2* ($n = 7$) groups.

Survival analysis. Cumulative survival of all familial breast cancer patients with no cytoplasmic HuR expression was 80.2% [95% confidence interval (95% CI), 74.9-85.5] and 69.9% (95% CI, 63.0-76.8) in patients with cytoplasm-positive HuR expression ($P = 0.0049$; Fig. 1A). When survival analyses were done separately in subgroups based on the *BRCA1/2* gene status, we found that among non-*BRCA1/2* mutation carriers, the cumulative survival was 82.0% (95% CI, 76.3-87.6) for the HuR cytoplasm-negative group and 73.7% (95% CI, 65.9-81.5, $P = 0.0257$; Fig. 1B) for the HuR cytoplasm-positive group. In the *BRCA1* group, the cumulative survival was 61.5% (95% CI, 38.9-84.1) for HuR cytoplasm-negative cases and 73.6% (95% CI, 55.9-91.1, $P = 0.2282$; Fig. 1C) for the HuR cytoplasm-positive cases. In the *BRCA2* group, the cumulative survival was 61.0% (95% CI, 29.7-92.3) for the HuR-negative cases and 47.3% (95% CI, 28.9-65.7, $P = 0.1544$; Fig. 1D) for the HuR cytoplasm-positive cases.

Multivariate analysis. To determine whether HuR expression could work as an independent prognostic factor in familial non-*BRCA1/2* cases, the stepwise Cox regression multivariate model was used. HuR expression was entered into a multivariate model together with lymph node status, tumor size,

Table 1. Histopathologic characteristics of familial non-BRCA1/2 breast cancer patients

Variable	*n* (%)	Cytoplasmic HuR immunopositivity, *n* (%)		*P**	OR (95% CI)
		Negative	Positive		
T					
T_1	321 (61.5)	197 (62.1)	124 (60.5)	NS	1.07 (0.75-1.54)
T_{2-4}	201 (38.5)	120 (37.9)	81 (39.5)		
N					
Negative	320 (59.0)	189 (57.6)	131 (61.2)	NS	0.86 (0.61-1.22)
Positive	222 (41.0)	139 (42.4)	83 (38.8)		
M					
Negative	522 (97.6)	320 (98.2)	202 (96.7)	NS	1.85 (0.61-5.58)
Positive	13 (2.4)	6 (1.8)	7 (3.3)		
ER					
Negative	111 (21.6)	48 (15.4)	63 (31.3)	<0.0001	0.40 (0.26-0.61)
Positive	402 (78.4)	264 (84.6)	138 (68.7)		
PR					
Negative	173 (34.1)	86 (27.9)	87 (43.5)	0.0003	0.50 (0.35-0.73)
Positive	335 (65.9)	222 (72.1)	113 (56.5)		
p53					
Negative	417 (78.2)	274 (84.3)	143 (68.8)	<0.0001	2.44 (1.63-3.71)
Positive	116 (21.8)	51 (15.7)	65 (31.3)		
Grade					
1	115 (22.3)	85 (27.1)	30 (14.9)	0.0001	NA
2	246 (47.7)	154 (49.0)	92 (45.5)		
3	155 (30.0)	75 (23.9)	80 (39.6)		
Histology					
Ductal	377 (67.0)	215 (63.4)	162 (72.3)	0.0216	NA
Lobular	92 (16.3)	67 (19.8)	25 (11.2)		
Medullary	8 (1.4)	3 (0.9)	5 (2.2)		
Other	86 (15.3)	54 (15.9)	32 (14.3)		

Abbreviations: OR, odds ratio; ER, estrogen receptor; PR, progesterone receptor; NS, nonsignificant; NA, not applicable.
*χ^2 test.

histologic grade, estrogen receptor and progesterone receptor status, and p53 immunopositivity. In this multivariate survival analysis, cytoplasmic HuR immunopositivity (P = 0.0311), tumor size (P = 0.0014), lymph node status (P = 0.0176), and histologic grade (P = 0.0029) were identified as independent prognostic factors (Table 2).

Discussion

We found cytoplasmic HuR protein expression to be an independent prognostic factor in non-*BRCA1/2* mutated hereditary breast cancer patients. This result is similar to our earlier study on sporadic invasive ductal breast cancers (18). We found cytoplasmic HuR expression in 39% of the non-*BRCA1/2* mutated tumors, which is slightly higher than presented in earlier reports on sporadic breast cancer (29% and 30%; refs. 18, 28). In non-*BRCA1/2* mutated hereditary breast cancers, cytoplasmic HuR immunopositivity associates with estrogen receptor and progesterone receptor negativity, p53 immunopositivity, high tumor grade, and ductal histology. We and others have previously observed similar associations of cytoplasmic HuR with tumor grade (18, 28) and p53 status[7] in sporadic breast cancers. These data suggest that HuR expression may have a similar role in tumorigenesis among familial non-*BRCA1/2* tumors as in sporadic cancers.

In *BRCA1/2* mutation carriers, cytoplasmic HuR expression was elevated, being 63% in *BRCA1* and 62% in *BRCA2* mutated groups. This is a relatively high frequency because it has previously been reported to range from 29% to 53% in breast, ovarian, gastric, and colorectal cancers (14–18, 28, 29). *BRCA2* patients with cytoplasmic HuR expression also showed a reduction in survival, but this result was not statistically significant. Among *BRCA1* mutation carriers, no such effect was observed, and, indeed, in the case of *BRCA1* it seems unlikely that an association to that in non-*BRCA1/2* cases would be found even in a larger material. However, the limited sample size in this analysis does not allow firm conclusions on a role of HuR in *BRCA1* or *BRCA2* tumors, and further studies are needed to evaluate the significance of HuR in *BRCA1* and *BRCA2* mutation carriers.

In *BRCA1* mutated cases, we found, similarly to non-*BRCA1/2* mutated cases, a correlation between cytoplasmic HuR expression and p53 positivity. Although p53 status did not reach statistical significance in *BRCA2* mutated tumors, all seven p53-positive tumors exhibited cytoplasmic HuR. Thus, association of p53 positivity with cytoplasmic HuR expression seems to be a general theme in breast cancer. HuR has been reported to regulate p53 by stabilizing its mRNA and enhancing its translation in rat intestinal epithelial cells and in human colorectal cancer cells (30, 31), which could explain this association. It is not known whether p53 activation or inactivation modulates cytoplasmic HuR content, but it is unlikely that this hypothetical association could alone explain increased cytoplasmic HuR levels, because the number of p53-positive cases (22%) in our material is relatively low when compared with cytoplasmic HuR-expressing tumors (43%).

[7] Our unpublished data.

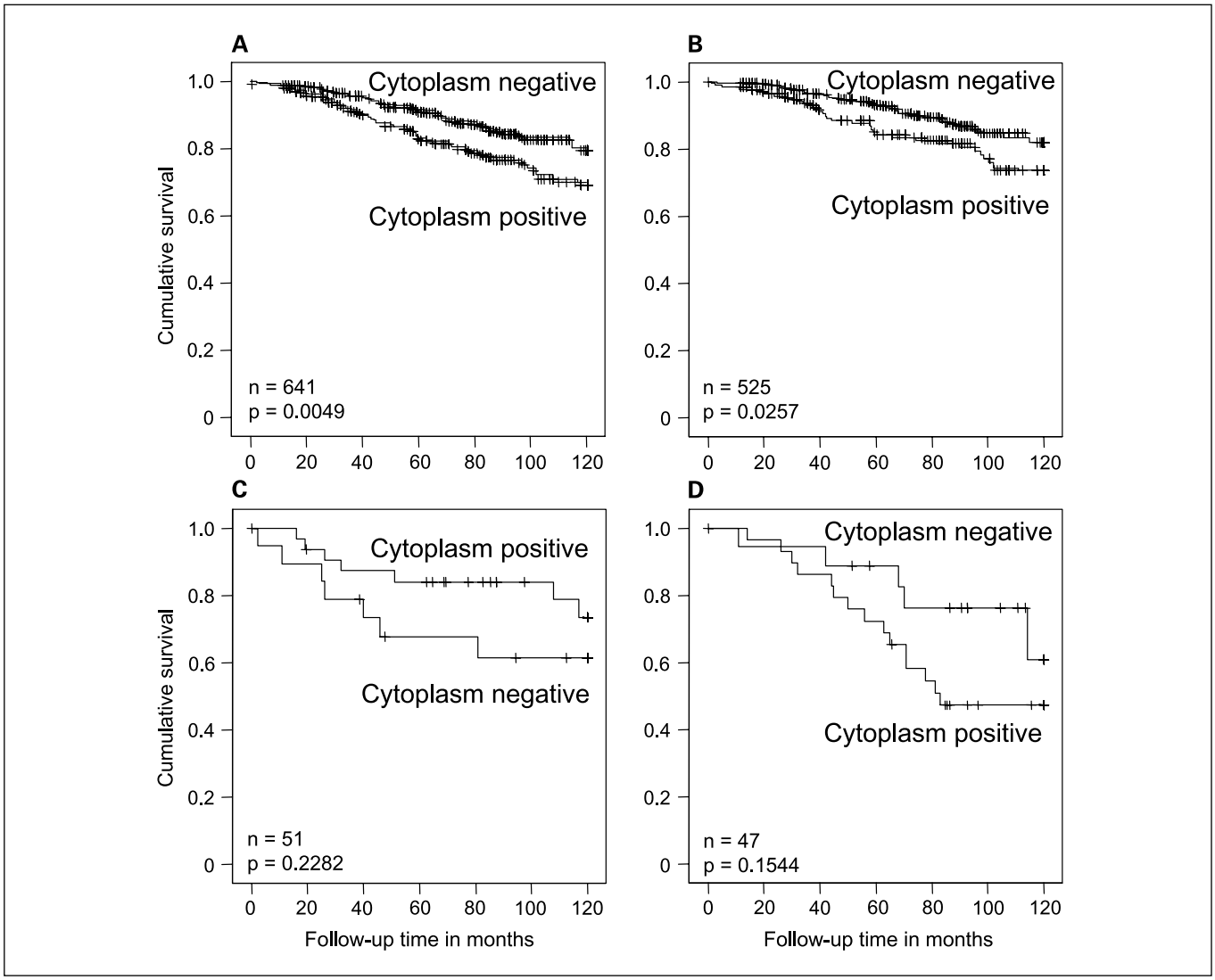

Fig. 1. Cumulative 10-y survival of familial breast cancer patients according to cytoplasmic HuR immunohistochemistry. *A,* all familial breast cancer patients. *B,* familial breast cancer patients with normal *BRCA1/2* gene status. *C,* familial breast cancer patients with mutated *BRCA1*. *D,* familial breast cancer patients with mutated *BRCA2*.

Mutations in *BRCA1/2* genes have been shown to cause increased chromosomal instability, which can lead to DNA damage response and cell stress (23). Cell stress responses can activate signal transduction pathways, such as mitogen-activated protein kinases in which activation of p38 has been shown to increase cytoplasmic HuR and enhance its binding to target mRNAs (7, 32), which include several gene products that participate in response to cell stress (6, 30, 31, 33). Recently, it was shown that HuR can be posttranslationally modified. Abdelmohsen et al. (34) showed that HuR is phosphorylated by cell cycle checkpoint kinase 2. Phosphorylation of HuR did not have an effect on its intracellular localization but rather regulates its binding to the target mRNA. All these results indicate that cell stress can cause increase in cytoplasmic HuR levels and alter the interaction with its target transcripts.

We show here for the first time that cytoplasmic HuR expression is an independent prognostic factor in familial breast cancer patients. Based on these data and our earlier study (18), HuR is a marker of poor prognosis among both sporadic and familial breast cancer patients, and may contribute to the carcinogenesis in this disease.

Table 2. Multivariate analysis of familial non-BRCA1/2 breast cancer patients

Variable	*P*	RR (95% CI)
Tumor size	0.0014	2.49 (1.42-4.34)
Nodal status	0.0176	1.89 (1.12-3.18)
Histologic grade	0.0029	1.88 (1.24-2.85)
HuR cytoplasmic	0.0311	1.74 (1.05-2.88)

Abbreviation: RR, relative risk.

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