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PRECLINICAL STUDY

Hyaluronan synthases (HAS1–3) in stromal and malignant cells correlate with breast cancer grade and predict patient survival

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Abstract Accumulation of hyaluronan (HA) in pericellular stroma and carcinoma cells is predictive of unfavorable patient prognosis in many epithelial cancers. However, it is not known whether the HA originates from carcinoma or stromal cells, or whether increased expression of hyaluronan synthase proteins (HAS1–3) contributes to HA accumulation. In this study, localization and expression of HAS1–3 were evaluated immunohistochemically in 278 cases of human breast cancer, and correlated with prognostic factors and patient outcome. Both carcinoma cells and stromal cells were HAS-positive. In carcinoma cells, HAS1 and HA stainings correlated with each other, and HAS1 associated with estrogen receptor negativity, HER2 positivity, high relapse rate, and short overall survival. In stromal cells, the staining levels of all HAS isoforms correlated with the stromal HA staining, stromal cell CD44, high relapse rate, and short overall survival of the patients. In addition, expression levels of stromal HAS1 and HAS2 were related to obesity, large tumor size, lymph node positivity, and estrogen receptor negativity. Thus, stromal HAS1 and HAS3 were independent prognostic factors in the multivariate analysis. The data suggest that increased levels of HAS enzymes contribute to the accumulation of HA in breast cancer, and that HA is synthesized in carcinoma cells and stromal cells. The study also indicates that HAS enzyme levels are related to tumor aggressiveness and poor patient outcome representing potential targets for therapy.

Keywords Breast cancer · Hyaluronan synthase · HER2 · Obesity

Abbreviations

HA	Hyaluronan
Has, HAS	Hyaluronan synthase gene and protein
bHABC	Biotinylated hyaluronan binding complex
HR	Hazard ratio
TSG-6	Tumor necrosis factor alpha stimulated gene-6
IαI	Inter alpha inhibitor
UDP	Uridine diphosphate
RAR	Retinoic acid receptor
STAT	Signal transducer and activator of transcription
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
CREB	cAMP response element binding protein
CISH	Chromogenic in situ hybridization
BMI	Body mass index

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GlcUA Glucuronic acid
GlcNAc *N*-acetyl-glucosamine

Introduction

Hyaluronan (HA) is a ubiquitous component of the extracellular matrix, present in the most normal tissues but its levels are markedly increased in many carcinomas [1]. In breast carcinoma, a high concentration of HA in pericellular stroma and carcinoma cells strongly associates with poor differentiation of tumors and unfavorable patient prognosis [2].

Hyaluronan is produced at the intracellular face of the plasma membrane by three isoforms of hyaluronan synthases (HAS1–3) [3]. The three HASs have different expression patterns and enzymatic properties [3]. However, the functional importance of the three enzymes is not entirely clear, since only *Has2* knockdown is embryonically lethal [4] while *Has1* and *Has3* deletions have only minor effects on the phenotype [5]. During HA synthesis, HAS transfers the alternating glucuronic acid (GlcUA) and *N*-Acetyl-glucosamine (GlcNAc) moieties from their respective UDP-derivatives into this glycosaminoglycan, simultaneously extruding the HA-chain into the extracellular space [3]. The newly synthesized HA is either released into the matrix or remains attached to the plasma membrane, retained there by either HAS or by the specific HA receptor, CD44, and thus forming a pericellular HA-coat [6, 7].

Regulation of HAS activity is complex and occurs at both the transcriptional and post-transcriptional levels [5]. Growth factors and cytokines are known to be major regulators of *Has* gene expression, especially those signaling through the ErbB, FGF, and TGFβ families [5]. Post-transcriptional modifications of HAS, such as addition of phosphate and *O*-GlcNAc moieties [5], as well as HAS transport to and residence within the plasma membrane strongly influence the enzymatic activity [8]. In addition, the cellular contents of the HAS substrates UDP-GlcUA and UDP-GlcNAc regulate HA synthesis [9].

Only a few clinical studies have focused on the presence and location of HAS isoenzymes in malignant human tumors. In bladder carcinoma, the level of HAS1 mRNA was significantly increased, and this elevation predicted metastasis [10]. The concentration of HAS1 mRNA was also higher in renal cell carcinomas as compared with normal tissue, but this was not the case in benign renal tumors [11]. In addition, increased HAS1 mRNA expression in colon cancer [12] and elevated HAS1 protein expression in ovarian cancer [13] have both correlated with poor survival of patients. While, it is thought that most of the HA accumulated in the tumor stroma is produced by HAS1–3 in the stromal cells [1], there are also reports of an increase in the amount of HAS protein present in carcinoma cells [14].

The distribution and amount of HAS1–3 proteins have not been studied in human breast carcinoma, although there are experimental studies on breast cancer cells demonstrating the importance of HAS2 as an enhancer of cancer cell invasiveness and aggressiveness [15]. It has been claimed that HAS2 has a critical role in the development of a prometastatic microenvironment [16]. The aim of this study was to investigate the localization and levels of HAS1–3 proteins in human breast carcinoma cases. Furthermore, tumoral HAS1–3 levels were correlated with those of CD44 and HA, as well as with a number of clinical parameters i.e. tumor size, nodal involvement, HER2, ER, tumor grade, relapse rate, and survival. A strong prognostic value for unfavorable outcome was associated with all HASs, especially with HAS1.

Patients and methods

The criteria for patient selection have been described previously [17]. Shortly, the material included 278 breast carcinoma cases, consisting of 139 HER2 positive and 139 HER2 negative cases matched with the time of the operation and the age of the patients. With the exception of the HER2-status, no other pathological and clinical data were reviewed before the selection of the patients. Permission for this study was provided by the ethics committee of the University of Eastern Finland, and also by the National Supervisor Authority for Welfare and Health (VALVIRA).

The baseline characteristics of the patients have been described previously [17]. Briefly, all of the 278 cases had invasive breast carcinoma, and in addition 98 patients (35 %) had also an in situ component. Two hundred and twenty-eight patients (82 %) had ductal, 27 (10 %) had lobular, 5 (2 %) mucinous, and 18 (6 %) presented with some other histological subtype. Seventy-four (27 %) of the patients had stage one, 153 (55 %) had stage 2, and 51 (18 %) were diagnosed with stage three disease. The mean age of the patients was 58 years (ranging from 32 to 86 years), and the mean follow-up time was 6.3 years (ranging from 0.4 to 11.1 years). As an adjuvant hormonal treatment 107 (39 %) received tamoxifen, 40 (14.5 %) were treated with an aromatase inhibitor, 23 (8 %) had switched from tamoxifen to aromatase inhibitor, and 4 (1.5 %) had Zoladex®. These were adjuvant chemotherapy regimens; 79 (28 %) received an anthracycline, 69 (25 %) anthracycline and taxane, 3 (1 %) only taxane therapy, and 53 (19 %) an adjuvant therapy based on cyclophosphamide, methotrexate,

and 5-fluorouracil (CMF). Adjuvant trastuzumab treatment was given to 63/139 (45 %) of the HER2-positive patients. Postoperative radiotherapy was provided to 248 (89 %) of all patients.

HAS, HA, and CD44 stainings

The deparaffinised sections were subjected to antigen retrieval by incubation in 10 mM citrate buffer, pH 6.0 for 15 min in a pressure cooker at 120 °C. In order to block endogenous peroxidase, the sections were treated for 5 min with 1 % H_2O_2. After washing with 0.1 M Na-phosphate buffer, pH 7.4 (PB), the sections were incubated in 1 % bovine serum albumin (BSA) and 0.1 % gelatin solution (Sigma G-2500, Sigma-Aldrich, MO) in PB for 30 min to block nonspecific binding. Hyaluronan synthases (HAS1–3) were detected by incubating the sections overnight at 4 °C with polyclonal antibodies for HAS1 (2 μg/ml, sc-34021, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HAS2 (2 μg/ml, sc-34067, Santa Cruz) or HAS3 (2 μg/ml sc-34204, Santa Cruz), diluted in 1 % BSA. After washed with PB, the sections were incubated for 1 h with biotinylated antigoat secondary antibody (1:1,000, Vector Laboratories) and visualized with the avidin–biotin peroxidase method (1:200, Vectastain Kit, Vector Laboratories) followed by incubation for 5 min in 0.05 % diaminobenzidine (Sigma) and 0.03 % hydrogen peroxide in PB, yielding a brown reaction product. The nuclei were stained with Mayer's hematoxylin. The specificity of the antibody stainings was controlled by preincubating the antibodies with the peptides used for immunization, which reduced the staining intensity with each antibody (Supplementary Fig. S1). The possible cross-reactivity between the antibodies against HAS1–3 has been controlled by staining MCF7 cells transfected with HAS1–3 constructs. No cross-reactivity was observed [18]. The staining methods used for HA and CD44, and their evaluation have been described before [17].

The evaluation of the HAS1–3

Hyaluronan synthase protein immunostainings were evaluated using representative 5 μm thick tumor sections. Several randomly picked high-power fields were evaluated in each section. The expression of HAS1–3 in breast carcinoma cells and stromal cells (i.e. fibroblasts, myofibroblasts and endothelial cells) was graded according to the percentage of positive cells: negative (0–5 %); weak (6–25 %); moderate (26–50 %); high (51–75 %); or very high (76–100 %). The sections were evaluated by three independent evaluators (YS, PA, RTu). In difficult cases, and those with discrepancy between the evaluators, the final decision was made by the leader of the group (YS).

Fluorescent co-staining of HAS2 and hyaluronan

Co-staining was done to compare the localization of HAS with that of HA in stromal cells. The HAS2 isoform was selected because it is known to be especially abundant in stromal cells.

Deparaffinised sections were treated with 50 mM glycine for 20 min at room temperature to quench any autofluorescence. The sections were blocked with 1 % bovine serum albumin for 30 min, followed by an overnight incubation with the primary antibody against HAS2 (2 μg/ml, sc-34067, Santa Cruz) together with 3 μg/ml of the biotinylated hyaluronan binding complex (bHABC), prepared in our laboratory [19]. After washing, the sections were incubated for 1 h with the secondary antibody, (1:500, Texas Red antigoat IgG, Vector) and streptavidin (1:500, FITC Streptavidin, Vector). Nuclei were labeled with DAPI (1 μg/ml, Sigma-Aldrich). The sections were mounted in Vectashield (Vector H-1000, Vector).

Statistical analyses

The statistical analyses were conducted using SPSS version 17.0. The data concerning follow-up time, relapses, deaths, and overall survival were obtained from hospital registers which are linked with the National Population Registry. The associations between staining intensities and clinical parameters were calculated with the Chi square test. The overall survival time was calculated from the date of diagnosis to the date of death. The univariate analyses for overall survival were conducted using the Kaplan–Meier method, and the significance of the differences between groups was assessed by the log-rank test. The multivariate analyses for survival were done using Cox's model, including HAS isoforms, tumor size, lymph node status, grade of the tumor, estrogen and progesterone receptor status, HER2 status, and trastuzumab therapy among the HER2-positive cases. This study was done according to the recommendation criteria in tumor marker prognostic studies (REMARK) [20].

Results

HAS1, HAS2, and HAS3 were all expressed very frequently in breast carcinoma cells, and they were mostly seen in the cytoplasm, and occasionally on the cell surface and nucleus (Fig. 1a–i). The distribution corresponded to the large intracellular pool of HASs in the endoplasmic reticulum-Golgi compartment and nuclear membrane [18]. More than half of the breast carcinoma cells were HAS1, HAS2, and HAS3 positive in 94, 99, and 92 % of the cases, respectively, and in none of the cases were all breast

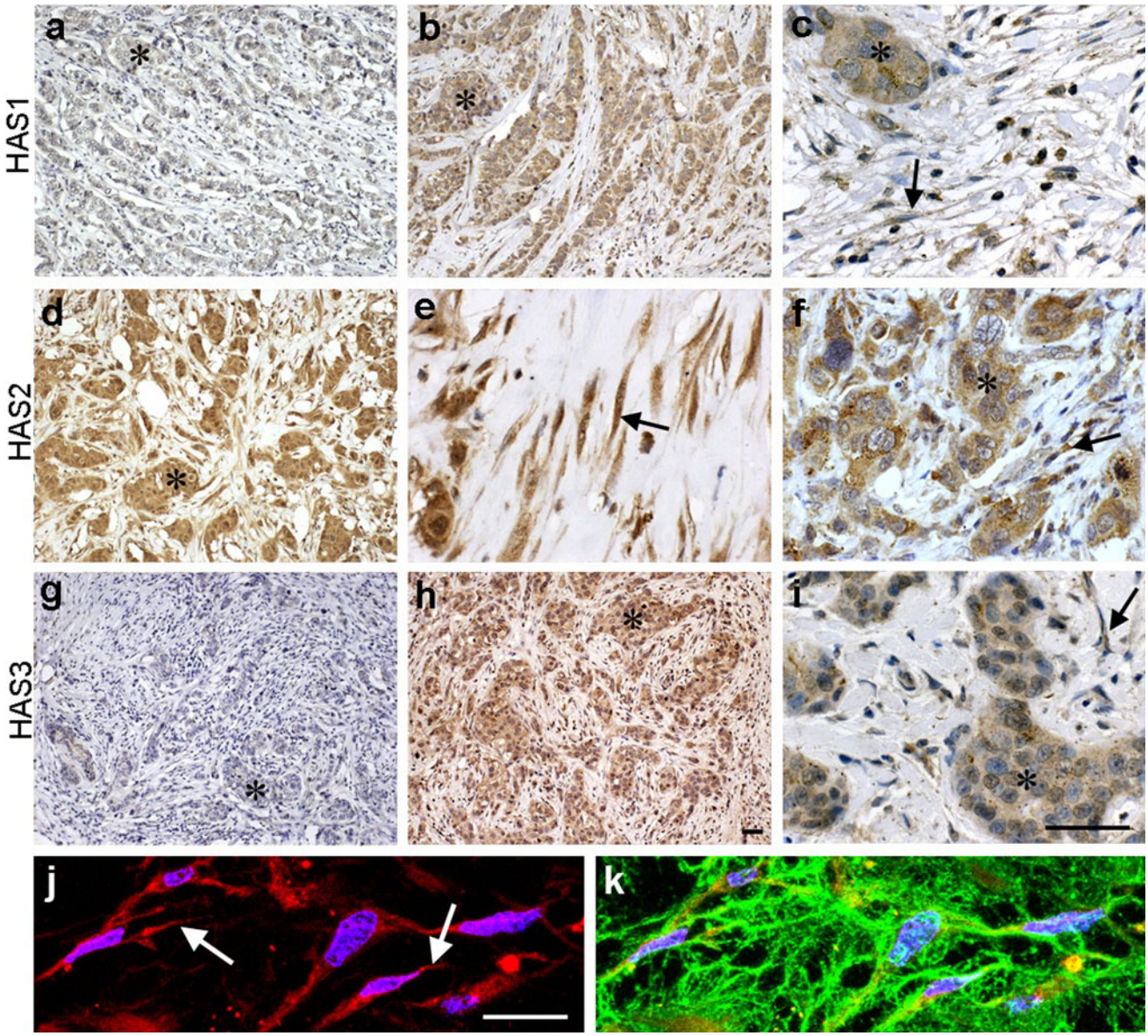

Fig. 1 Hyaluronan synthase isoenzymes in breast carcinoma sections. HAS1 staining is shown in (**a**–**c**), HAS2 in (**d**–**f**), and HAS3 in (**g**–**i**). An overview of low (**a**) and high (**b**) levels of HAS1 staining in tumor and stromal cells, and at higher magnification in (**c**). **d** shows an overview of strong HAS2 staining, and **e**, **f** higher magnification of HAS2 staining in stromal and tumor cells, respectively. Mostly negative and strong areas of HAS3 staining are shown in **g** and **h**, respectively, and a higher magnification in (**i**). Tumor cells are marked by *asterisks*, stromal cells by *arrows*. The co-localization of HAS2 (*red*) and HA (*green*) in dual staining are shown in **j**, **k**. HAS2 is shown in **j**, and together with HA in **k**. Nuclei are marked with *blue* color. *Arrows* in **j** indicate HAS-positive cell protrusion. *Magnification bar* for low magnification (50 μm) (**a**, **b**, **d**, **g**, **h**) is presented in **h**, and high magnification (20 μm) (**c**, **e**, **f**, **i**) in **i**. *Magnification bar* (20 μm) for fluorescent images is shown in **j**

carcinoma cells completely negative for HAS1, HAS2 or HAS3 immunoreactivity (Fig. 2a).

HAS1, HAS2, and HAS3 were also detected in all types of stromal cells (i.e. fibroblasts, myofibroblasts, and endothelial cells) (Fig. 1a–i). The proportion of HAS-positivity was generally lower in stromal cells than in carcinoma cells. 64 (23 %) and 44 (16 %) of the samples were totally negative for stromal cell-associated HAS1 and HAS3, respectively, while a mere 3 (1 %) were negative for HAS2 (Fig. 2b). More than half of the stromal cells were positive for HAS1, HAS2, and HAS3 in 13, 52, and 15 % of cases, respectively (Fig. 2b).

The association of HAS1–3 with HA and CD44

In a Chi square tests, a high percentage of HAS1 positive carcinoma cells correlated with a high percentage of HA positive carcinoma cells (HR 35.06, $p < 0.001$). Both

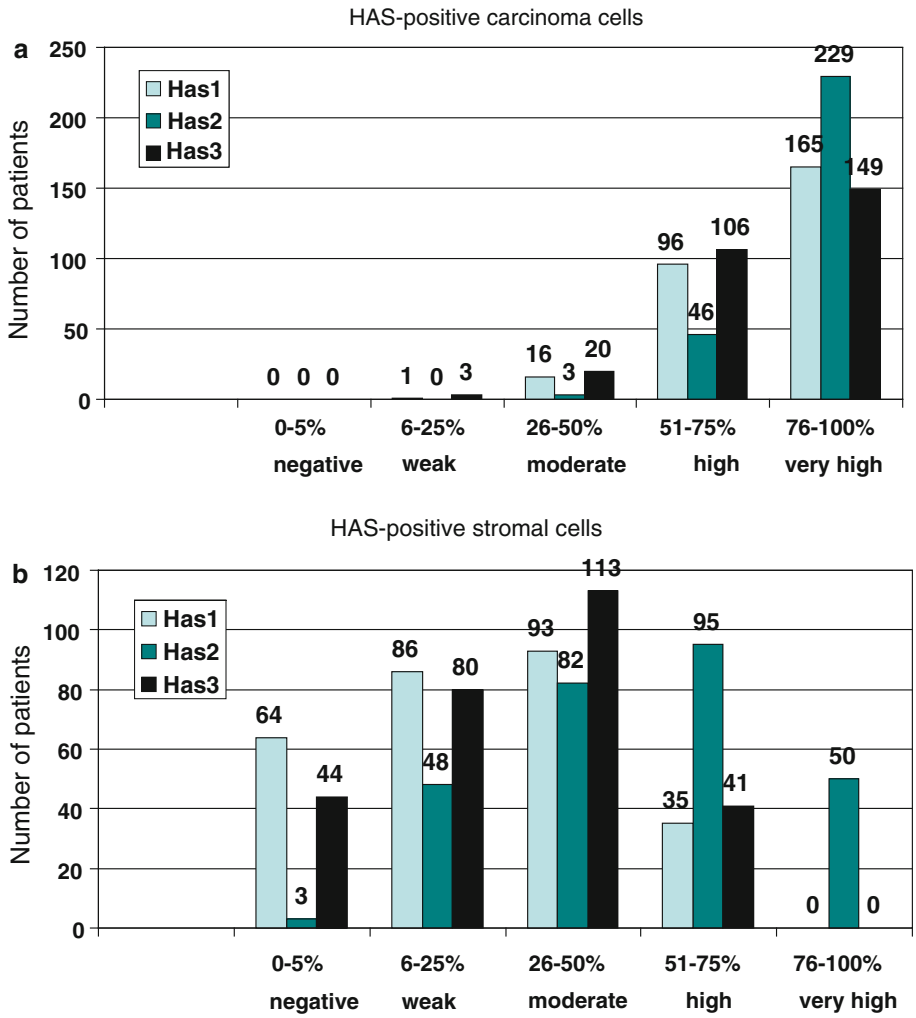

Fig. 2 The proportion of cells positive for HAS1–3 in 278 human breast carcinoma lesions. **a** Breast carcinoma cells showed a high percentage of HAS1, HAS2, and HAS3 positivity. **b** In stromal cells, HASs were not expressed as often as in carcinoma cells, HAS2 being the most frequently expressed isoform

HAS1 and HAS2 expression levels in carcinoma cells correlated with a high stromal HA level ($p < 0.01$; Table 1). The immunoreactivities of all HASs in the carcinoma cells correlated with CD44 positivity in stromal cells ($p < 0.005$), but not with CD44 positivity in carcinoma cells (Table 1).

All HAS isoforms in stromal cells correlated with a high level of stromal and carcinoma cell-associated HA ($p < 0.001$), and also with CD44 positivity in stromal cells ($p < 0.001$) (Table 1). Stromal cell HAS1 and HAS2 expression levels correlated also with CD44 in carcinoma cells ($p < 0.02$).

To visualize the co-distribution of HA and HASs in human breast cancer cases, we performed dual staining of HA and HAS2 in 5 cases, shown in Fig. 1j, k. HA was widely distributed in the stroma surrounding the HAS2-positive fibroblasts. Prominent HAS2 staining aligned with the plasma membrane of fibroblasts, including the cell processes extending to the stromal matrix.

The association of HAS1–3 with prognostic factors

High immunoreactivities for HAS1, HAS2, and HAS3 in carcinoma cells were associated with poor differentiation of the tumor, but not with tumor size or lymph node positivity (Table 2 and Supplementary Tables S1–3). In addition, a high expression level of carcinoma cell-associated HAS1 was related to HER2 positivity, since 63.6 % of the patients with very high HAS1 expression levels in carcinoma cells were HER2 positive (HR 31.56, $p < 0.001$). There was also an association between a high HAS1 expression level in carcinoma cells and estrogen receptor negativity (HR 10.02, $p = 0.018$) (Table 2 and Supplementary Tables S1–3).

High percentages of HAS1-, HAS2-, and HAS3-positive stromal cells were associated with large tumor size ($p < 0.001$) and poor differentiation (i.e. high grade) of the tumor ($p < 0.001$) (Table 2 and Supplementary Tables S4–6). In addition, high percentages of HAS1 and HAS2

Table 1 The correlation of HAS1–3 with HA and CD44

Factor	HA in carcinoma cells	HA in stromal matrix	CD44 in carcinoma cells	CD44 in stromal cells
HAS1 in carcinoma cells	$p < 0.001$	$p < 0.001$	ns	$p < 0.001$
HAS2 in carcinoma cells	ns	$p = 0.009$	ns	$p = 0.004$
HAS3 in carcinoma cells	ns	ns	ns	$p = 0.003$
HAS1 in stromal cells	$p < 0.001$	$p < 0.001$	$p = 0.006$	$p < 0.001$
HAS2 in stromal cells	$p < 0.001$	$p < 0.001$	$p = 0.012$	$p < 0.001$
HAS3 in stromal cells	$p < 0.001$	$p < 0.001$	ns	$p < 0.001$

positivity in the stromal cells were associated with both lymph node positivity ($p < 0.015$) and estrogen receptor negativity ($p < 0.045$). A high percentage of HAS2 positive stromal cells was associated with HER2 positivity (HR 14.40, $p = 0.006$) (Table 2 and Supplementary Tables S4–6).

The association of HAS1–3 with the weight and BMI of the patients

When comparing HAS immunoreactivities with patient body weight, more than half of the stromal cells were HAS1 positive in 5/71 (7 %), 16/130 (12 %), and 14/77 (18 %) of the patient groups with weights ≤60, 61–75, and >75 kg, respectively (HR 13.25, $p = 0.040$). Similarly, over half of the stromal cells were HAS2 positive in 27/71 (38 %), 73/130 (56 %), and 45/77 (58 %) in the patient groups with weights ≤60, 61–75, and >75 kg, respectively (HR 15.35, $p = 0.05$). In addition, double (32 %) the number of the patients with high HAS3 expression level in stromal cells were obese (BMI ≥ 30) compared to the patients (16 %) with negative HAS3 expression level in stromal cells (HR 21.11, $p = 0.049$).

HAS expressions in comparison to relapses

In the whole patient group, 61/278 (22 %) had suffered a relapse during the follow-up time. The high levels of carcinoma cell-associated HAS1 expressions were related to an increased relapse rate, as 0, 6.5, 13.5, and 28.5 % of the patients suffered a relapse in weak, moderate, high, and very high expression level of HAS1 in carcinoma cells, respectively (HR 10.66, $p = 0.014$) (Table 3). The HAS1 expression in stromal cells was even more significantly associated with the relapses as 4.7, 16.3, 28, and 51.4 % of the patients with negative, weak, moderate, and high HAS1 expression in stromal cells suffered a relapse, respectively (HR 32.46, $p < 0.001$). Stromal cell-associated HAS2 (HR 24.62, $p < 0.001$) and HAS3 (HR 32.98, $p < 0.001$) expressions also correlated with the relapses (Table 3).

Relapses among the HER2-negative cases

In the HER2-negative cases, 16/139 (12 %) suffered a relapse. High percentages of HAS1, HAS2, and HAS3 in stromal cells were all associated with an increased relapse rate in this patient group. The statistically strongest correlation was detected between stromal cell HAS1, as 3, 5, 21, and 33 % of the patients suffered a relapse in the HER2-negative patient group with negative, weak, moderate, and high HAS1 level, respectively (HR 32.46, $p = 0.003$). Cancer cell-associated HASs were not related to relapse rate among the HER2-negative patients.

HER2-positive cases

In the HER2-positive cases, 45/139 (32 %) experienced a relapse, and there were significantly more relapses among trastuzumab untreated 32/76 (42 %) than among the trastuzumab treated 13/63 (21 %) patients (HR 7.25, $p = 0.007$). In the HER2-positive patient group, stromal cell-associated HAS1 (HR 15.71, $p = 0.001$), HAS2 (HR 9.67, $p = 0.046$), and HAS3 (HR 17.77, $p = 0.001$)

Table 2 The correlation of HAS1–3 with the clinical factors

	Tumor size	Nodal status	ER	Grade	HER2
HAS1 in carcinoma cells	ns	ns	$p = 0.018$	$p = 0.005$	$p < 0.001$
HAS2 in carcinoma cells	ns	ns	ns	$p = 0.004$	ns
HAS3 in carcinoma cells	ns	ns	ns	$p = 0.007$	ns
HAS1 in stromal cells	$p = 0.007$	$p = 0.01$	$p = 0.015$	$p = 0.001$	ns
HAS2 in stromal cells	$p = 0.035$	$p = 0.013$	$p = 0.043$	$p = 0.003$	$p = 0.006$
HAS3 in stromal cells	$p = 0.001$	ns	ns	$p = 0.001$	ns

Table 3 The relapse rate of the patients according to the HAS expressions

	The expression level of HASs					
	0–5 (%)	6–25 (%)	26–50 (%)	51–75 (%)	76–100 (%)	*p* value
HAS1 in carcinoma cells	No cases	0 (0 %)	1 (6 %)	13 (14 %)	47 (29 %)	$p = 0.014$
HAS2 in carcinoma cells	No cases	No cases	0 (0 %)	7 (15 %)	54 (24 %)	ns
HAS3 in carcinoma cells	No cases	0 (0 %)	5 (25 %)	22 (21 %)	34 (23 %)	ns
HAS1 in stromal cells	3 (5 %)	14 (16 %)	26 (28 %)	18 (51 %)	No cases	$p < 0.001$
HAS2 in stromal cells	0 (0 %)	2 (4 %)	15 (18 %)	22 (23 %)	22 (44 %)	$p < 0.001$
HAS3 in stromal cells	2 (5 %)	10 (13 %)	28 (25 %)	21 (34 %)	No cases	$p < 0.001$

HAS hyaluronan synthase, *ER* estrogen receptor, *HER2* human epidermal growth factor 2, *ns* non significant, *No cases* there are no cases at all in the group, *value 0* there are cases, but not relapses in the group

expressions associated with relapses, while carcinoma cell-associated did not show any association.

In the trastuzumab untreated patients, the stromal cell expression level of HAS3 was related to relapses as 9, 32, 46, and 77 % patients with negative, weak, moderate, and high HAS3 expression levels in stromal cells suffered a relapse (HR 12.39, $p = 0.006$). The stromal cell HAS1 expression level was also related to relapses in this patient group (HR 10.84, $p = 0.013$).

Only 13 relapses have occurred among the 63 trastuzumab-treated HER2 positive patients, precluding reliable statistical analysis because of the small number of cases. However, all the 13 relapses were among the cases displaying over 75 % of the carcinoma cells as HAS1 positive.

Survival analysis

If one considers the three isoenzymes, then HAS1 exhibited the strongest correlation with the outcome of the patients. While HAS2 and HAS3 positivity in breast carcinoma cells did not correlate with survival, a high level of HAS1 in carcinoma cells was associated with a short overall patient survival, since 77, 85, 94, and 100 of the patients with very high, high, moderate, and weak HAS1 expression in carcinoma cells were alive ($p = 0.024$) (Fig. 3a). In stromal cells, all HAS isoforms were related to short overall survival of the patients in the univariate survival analysis ($p < 0.001$) (Fig. 3b–d). In the HER2-positive patients, a high level of HAS1 expression in stromal cells was associated with a poor outcome of the patients as 44, 75, 76, and 88 % of the patients with high, moderate, weak, and negative HAS1 expression in stromal cells were alive, respectively ($p < 0.0001$). In addition, high HAS3 expression in stromal cells was associated with short survival of the HER2-positive patients ($p = 0.012$) (data not shown).

In the multivariate analysis of overall survival in the whole study group, stromal cell HAS1 (reference stromal cell HAS1: negative; stromal cell HAS1: 6–25 %, HR 1.485, $p = 0.223$; stromal cell HAS1: 26–50 %, HR 1.670, $p = 0.196$; stromal cell HAS1: 50–75 %, HR 9.434, $p = 0.002$), tumor size ($p = 0.041$), nodal status ($p < 0.001$), and estrogen receptor status ($p = 0.009$) were all significant unfavorable prognostic factors. When the multivariate analysis for overall survival was conducted for HER2-positive patients, HAS1 in stromal cells (reference stromal cell HAS1: negative; stromal cell HAS1: 6–25 %, HR 0.828, $p = 0.363$; stromal cell HAS1: 26–50 %, HR 0.684, $p = 0.408$; stromal cell HAS1: 50–75 %, HR 5.881, $p = 0.015$) together with estrogen receptor status ($p = 0.013$) and adjuvant trastuzumab treatment ($p = 0.005$) were the significant co-factors for unfavorable prognosis. In the HER2-negative patients, HAS3 in stromal cells (reference stromal cell HAS3: negative; stromal cell HAS3: 6–25 %, HR 0.002, $p = 0.969$; stromal cell HAS3: 26–50 %, HR 4.182, $p = 0.041$; stromal cell HAS3: 50–75 %, HR 1.962, $p = 0.161$), together with lymph node status ($p = 0.032$) were significant co-factors.

Discussion

The present study evaluated 278 breast cancer cases, and revealed for the first time that high levels of HAS1, HAS2, and HAS3 in stromal cells, and HAS1 in carcinoma cells, have clinical significance, since the expression levels of all of these enzymes were associated with disease recurrence and poor outcome of the patients. In addition, we detected strong correlations between HA and HAS1–3 immunoreactivities, suggesting that the accumulation of HA in breast cancer tissue is at least partly due to the increased expression levels of HAS1–3 proteins.

All HAS isoforms were widely expressed by both carcinoma and stromal cells. Scoring their immunoreactivity revealed that HAS1 was the only enzyme with prognostic significance in carcinoma cells, and in stromal cells, it was the strongest in predicting relapses and poor prognosis. This is partly an unexpected result, because HAS2 and

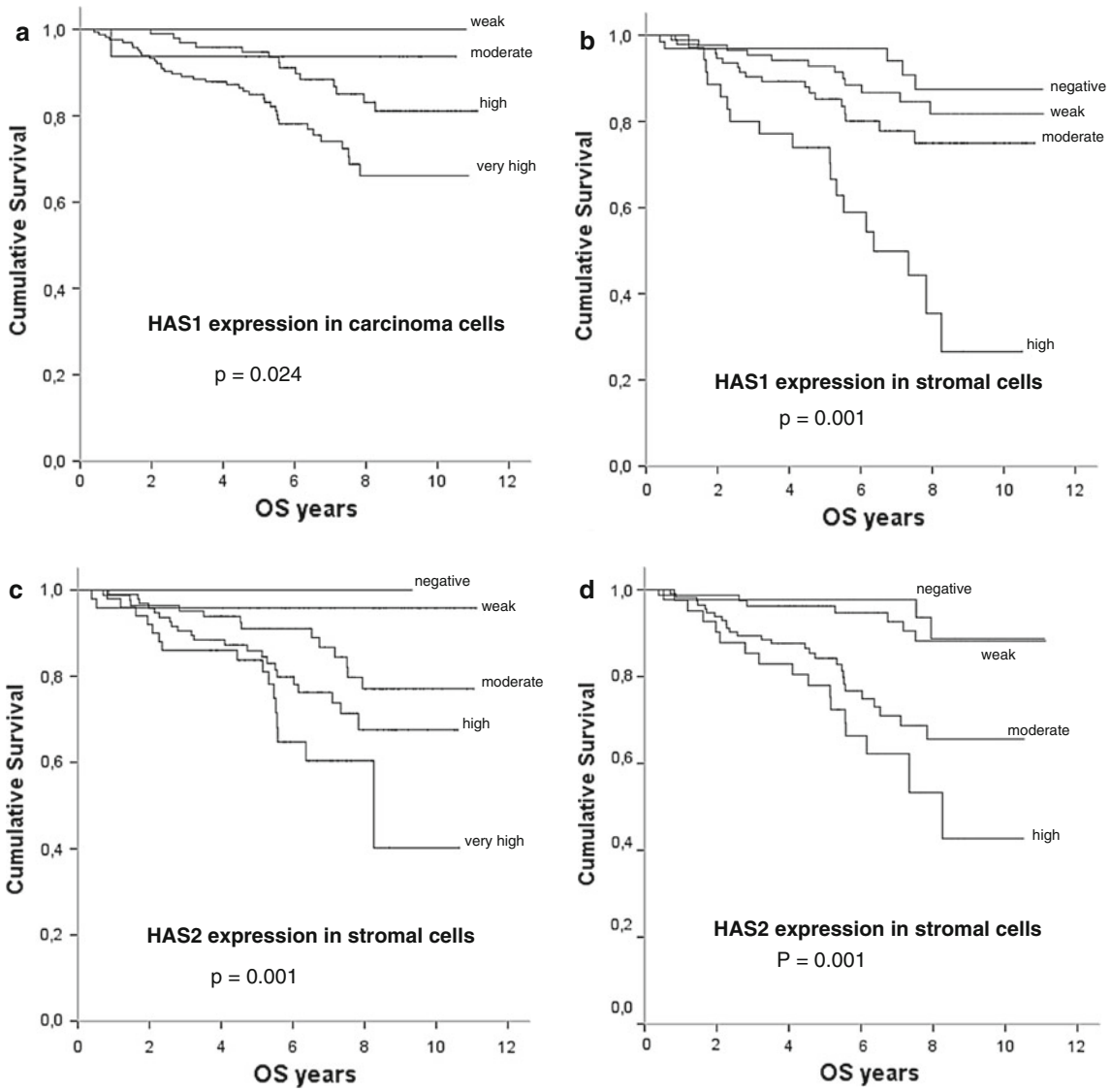

Fig. 3 Univariate analyses for overall survival (OS) according to the expression of HAS isoforms. **a** In carcinoma cells, only HAS1 was associated with the survival of the patients. **b–d** In stromal cells, all HAS isoforms were associated with OS of the patients

HAS3 have been proposed to be the most important isoforms in many pathological conditions [21, 22]. However, high expression levels of *Has1* mRNA have been associated with unfavorable prognosis in human colon cancer [12] and bladder cancer [10], and increased HAS1 immunoreactivity strongly associated with a poor outcome of the ovarian cancer patients [13]. Furthermore, overexpression of *Has1* gene has been reported to enhance the metastatic potential of breast cancer cells [23] and melanoma cells [24], pointing to an important role of *Has1* in malignant progression of cells. Nevertheless, it is difficult to define the roles of individual HAS enzymes in tumor promotion, since they are mostly expressed together, and may even function as heteromeric combinations [25]. In addition, the level of HAS2 protein in breast carcinoma cells was high in most patients, reducing the differences between the cases and, as a consequence, the power of statistical evaluation. Thus, the results of this study do not preclude the possibility that the HAS2 protein level is of significance in breast carcinoma cells.

The increase in the expression levels of HASs in stromal cells was more important for tumor progression and patient survival than the corresponding levels in carcinoma cells. This is in line with our previous reports [17] showing that especially stromal HA is significant for patient prognosis. A high HA content in the stroma surrounding tumor cells is believed to create a favorable niche for growth and spread of malignant cells, leading to invasion, metastasis and thus to poor prognosis [1]. Peritumoral stromal cells in breast cancer may actually be derived from the malignant

epithelial cells that have undergone epithelial-mesenchymal transition (EMT) [26]. In line with this hypothesis, *Has2* overexpression in mammary epithelial cells results in the cells acquiring mesenchymal characteristics [27] and *Has2* has a critical role in the TGFβ-induced EMT of mammary cells [28]. On the other hand, HA secreted by *Has2* overexpressing mammary tumor cells accelerates angiogenesis through the activation of stromal cells [29].

The present data suggests that HAS1–3 levels in stromal cells might be associated with a high body weight and obesity in the patients. In our previous study, a high stromal HA content was also associated positively with a high body mass index (BMI) [17]. Our study material is still too small to permit exhaustive statistical analyses, but these results are especially interesting, because in our other studies we have found that not only HAS2, but particularly HAS1, is more active in cells with a rich glucose and UDP-*N*-acetyl glucosamine (UDP-GlcNAc) supply [30]. Moreover, there is one report that HAS2 protein is stabilized in the presence of a high cellular UDP-GlcNAc content [31]. It is thus possible that the deranged glucose metabolism in cancer may be linked to the enhanced HA synthesis and HA content in malignant tumors, and the known fact that obesity is a breast cancer risk factor [32]. In support of this idea, a high peridiagnostic fasting blood glucose level and high BMI have both been associated with a high risk of breast cancer relapse rate and poor outcome in the patients [33].

Accumulation of hyaluronan metabolism is rather complex, with numerous signals and molecules associated with its synthesis and degradation, these being regulated at multiple levels. In addition to glucose metabolism, growth factor signaling is one of the best characterized stimulators of HA synthesis [5]. In our previous study, the content of HA was higher in HER2-positive patients, and the prognostic role of HA was most significant among the HER2-positive patients [17]. Accordingly, in the current study, HAS expression was strongly associated with HER2 positivity, and among HER2-positive patients the HAS expressions associated with the outcome of the patients also in the multivariate survival analysis. The present data thus further imply a connection between HER2 signaling and HA synthesis, and suggest that the high expression level of HA on the cell surface and pericellular stroma may affect binding of trastuzumab to its receptor [34], and thus contribute to the bleak prognosis of HER2-positive patients.

The expression of the HA receptor CD44 was strongly associated with the levels of all HASs in stromal cells. Increased HA synthesis and CD44 expression are important factors in inflammatory reactions [35, 36], and the importance of the inflammatory component in the initiation and progression of cancer has become evident [37]. The finding of Baek et al. [38] that high serum concentration of CD44 is a predictor for a short overall survival, especially among the HER2-positive breast cancer patients, fits very well with our observations that the high expression level of CD44 in stromal cells associated with HAS expressions, HA content, and unfavorable prognosis. The CD44-HA axis likely recruit tumor inflammatory cells and modifies their functions to support malignant growth.

In summary, this work demonstrates that the high levels of HAS proteins, both in stromal and carcinoma cells, are associated with poor differentiation, HER2-positivity, and poor patient outcome. These findings are in line with many in vitro studies showing a correlation between high HAS expression, altered HA biosynthesis, and malignant properties of carcinoma cells [22]. Accordingly, in experimental animal studies, inhibition of HA synthesis suppresses growth and motility of breast carcinoma cells, and inhibits their expansion in osteolytic bone metastases [39]. The results of this study suggest that HASs are also clinically significant factors in the progression of breast cancer and thus potential targets for therapy.

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